



UNIVERSITY OF NAPLES FEDERICO II

**PhD in
MOLECULAR MEDICINE AND MEDICAL
BIOTECHNOLOGY**

XXX CYCLE

Rosario Avolio

Characterization of novel RNA-binding proteins in cancer



ACADEMIC YEAR 2016/2017

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ACADEMIC YEAR 2016/2017

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LIST OF ABBREVIATIONS

RBPs: RNA-binding proteins
RBDs: RNA-binding domains
mRBPs: messenger RNA-binding proteins
RRM: RNA recognition motif
KH: K homology
DSRM: double-stranded RNA-binding motif
Puf: Pumilio
snRNA: small nuclear RNA
snoRNA: small nucleolar RNA
ncRNA: non-coding RNA
QKI: Quaking
IGF2BP: insulin-like growth factor 2 mRNA binding proteins
HuR: Hu-antigen R
LARP3: La ribonucleoprotein domain family member 3
UNR: upstream of N-Ras
SR: serine/arginine-rich
STAR: signal transduction and activators of RNA
APA: alternative polyadenylation
3'-UTR: 3' untranslated region
CPEB: cytoplasmic polyadenylation element binding
TPA: tissue plasminogen activator
VEGF: vascular endothelial growth factor
ARE: AU-rich element
AUBPs: ARE-binding proteins
IRES: internal ribosome entry site
EMT: epithelial to mesenchymal transition
IMP1: insulin-like growth factor 2 mRNA binding protein 1
ACTB: actin B
CLIPs: Chaperones Linked to Protein synthesis
UPS: ubiquitin-proteasome system
TRAP1: tumour necrosis factor receptor-associated protein 1
HSP90: heat shock protein 90
LC-MS/MS: Liquid chromatography tandem mass spectrometry
ER: endoplasmic reticulum

PLA: proximity ligation assay
SDOS: Protein Syndesmos
53BP1: P53-binding protein 1
ATCC: American Type Culture Collection
FITR: Flp In T-Rex
PAR-CL: photoactivatable ribonucleoside-enhanced cross-linked
IP: immunoprecipitation
MITO: mitochondrial
CYTO: cytosolic
CHX: cycloheximide
mRNPs: messenger ribonucleoproteins
NaAsO₂: sodium arsenite
IF: immunofluorescence
PABP1: polyA binding protein 1
SGs: stress granules
WB: western blot
PNK: polynucleotide kinase assay
iCLIP: individual nucleotide resolution cross-linking and immunoprecipitation
RP: Ribosome Profiling
GE: Gene Expression
FDR: False Discovery Rate
GO: Gene Ontology
JBS: Joubert Syndrome
RPFs: Ribosome Protected Fragments
DEGs: Differentially Expressed Genes
DDR: DNA-damage response
PTMs: post-translational modifications

ABSTRACT

RNA-binding proteins (RBPs) are key players in the post-transcriptional regulation of gene expression, regulating each step of RNA metabolism, from synthesis to decay through a dynamic association. Accordingly, the repertoire of new non-canonical RBPs has consistently grown in the last few years. Given that post-transcriptional events play pivotal roles in the adaption of cells to the local microenvironment, it is common that perturbations of RBP-networks can lead to cancer through mechanisms that are still poorly understood. In this context, we investigated the role played by TRAP1, a molecular chaperone whose role in cancer has been extensively described, and its predicted interacting-partner Protein Syndesmos (SDOS). SDOS, also known as Nudt16l1, is a paralog of the catalytic nuclear Nudt16p family of proteins that has been predicted to lack the decapping activity. This work demonstrates that SDOS interacts with TRAP1, as shown by co-immunoprecipitation and proximity ligation assays. Moreover, SDOS associates with actively translating polyribosomes and takes part to stress granules, being involved in the downmodulation of mRNA translation. By both polynucleotide kinase (PNK) assay and small-scale RNA interactome capture, we demonstrated, for the first time, that SDOS and TRAP1 are novel, non-canonical RBPs. Consequently, we have characterized the RNA-binding properties of SDOS and TRAP1, by combining three high-throughput approaches: i) individual nucleotide cross-linking and immunoprecipitation (iCLIP) sequencing, to identify direct RNA targets; ii) Ribosome profiling sequencing, to identify differentially translated targets and iii) Gene expression analysis, to identify differentially expressed genes. Combination of these analyses allowed us to identify several crucial regulated pathways and, among those, we focused our attention on a small subset of genes responsible for ciliopathies, a class of rare diseases caused by defects in primary cilia. Among them we confirmed TMEM107, a ciliary transition zone protein, as directly bound at RNA level by SDOS, as demonstrated by RNA-immunoprecipitation analysis. Moreover, TMEM107 translational regulation by SDOS was demonstrated by western blot and qPCR assays. Taken together these findings suggest that SDOS might regulate primary cilia formation. Intriguingly, a new area of

research is emerging linking cilia to cancer, suggesting the existence of a bridge between SDOS and TRAP1 functions and related diseases.

1. INTRODUCTION

1.1. RNA-binding proteins

RNA-binding proteins (RBPs) represent about 7.5% of the eukaryotic proteome, belonging to a group of conserved, abundant, and ubiquitously expressed proteins, mostly involved in the post-transcriptional regulation of gene expression¹. These proteins bind specific regions of mRNAs, regulating each step of their life cycle, from synthesis to decay. RBPs recognise and bind short stretches of RNA due to the presence of specific amino acid motifs in their structure, therefore known as RNA-binding domains (RBDs)² - even though recent studies demonstrated that this statement is not entirely true, with novel identified RBPs lacking the classical RBDs. Although the multiple roles played by RBPs would suggest a large diversity in the structures responsible for the RNA recognition, most RBPs are actually built from a small number of RNA-binding modules. Thus, the recognition of a large diversity of substrates is ensured by the combination of multiple copies of these RBDs, that function together as a single RNA recognition unit³. Recently, it has been published a census of 1542 human RBPs which contain a repertoire of ~600 structurally distinct RBDs. Among the others, messenger RNA-binding proteins (mRBPs), are the most abundant across the different RBDs-based classes of RBPs. About 405 of the 692 mRBPs identified contain an RNA recognition motif (RRM), a K homology (KH) domain, a DEAD motif, a double-stranded RNA-binding motif (DSRM) or a zinc-finger domain, which are among the most well-known and characterized domains. Conversely, ribosomal proteins have 119 distinct domains, and they are exclusively found in this protein family¹. Messenger RBD classes usually occur in multiple repeats or in combination with other RBDs; for example, RRMs, KH domains, zinc-finger domains or cold-shock domains recognize a 4–6-nucleotide mRNA sequence and predominantly occur in combinations or repeats, thus increasing sequence specificity and affinity of RBPs; Pumilio (Puf) motif binds one nucleotide, staking of this motif into a domain allows the binding of 8 nucleotides³. A list of the most common RBDs identified so far is reported in Table 1.

Domain	Description
RRM	RNA recognition motif, single-strand RNA (ssRNA)-binding
RG/RGG	RG/RGG box repeats are arginine glycine rich low complexity regions, may bind RNA or act as protein-protein interaction domains in shuttling
DEAD	DEAD and DEAH box helicase motif, unwinds RNA (and DNA)
z1-COCH	Zinc finger motif type C-x8-C-x5-C-x3-H, ssRNA-binding
KH	KH-homology domain, ssRNA-binding
GTP_EFTU, GTP_EFTU_D2, GTP_EFTU_D3	GTP-elongation factor family, proteins usually consist of 3 structural domains, 2 oligonucleotide binding domains (D2 and D3) and a GTP-binding domain
dsm	Double-stranded RNA binding motif
z1-COHC	Zinc knuckle, C-x2-C-x4-H-x4-C, ssRNA-binding
LSM	Like Sm domain is found in snRNP complexes, bind AAU rich regions
OB_NTP_bind	Oligonucleotide/oligosaccharide-binding (OB)-fold, found in DEAD-box helicases in association with HA2 domain, regulates helicase activity through RNA binding
HA2	Helicase-associated domain, found in RNA helicases
G-patch	G-patch domain, ~48 amino acids with 6 conserved glycines, found in RBPs
IBN_N	Importin-beta N-terminal domain, RNA transport or RBP transport proteins
SAP	(SAF-A/B, Acinus and PIAS) motif, RNA/DNA-binding domain
TUDOR	Tudor domain, found in Tudor proteins, Tudor proteins are in complexes with RBPs
RnaseA	RNase A domain, ssRNA endonuclease
z1-C2H2_jaz	JAZ dsRNA-binding protein zinc-fingers, dsRNA-binding
MMR_HSR1	50S ribosome-binding GTPase domain, found in RBPs
KOW	KOW (Kyprides, Ouzounis, Woese) motif, found in a variety of ribosomal proteins
RNase_T	RNase T ssRNA exonuclease domain
MIF4G	MIF4G (Middle domain of eukaryotic initiation factor 4G (eIF4G)), RNA- (and DNA-) binding
z1-RanBP	RNA-binding Ran-binding-protein-like zinc finger
NTF2	Nuclear transport factor 2 (NTF2) domain, found in RNA export factors
PAZ	Piwi Argonaute and Zwiile (PAZ) domain, posttranscriptional silencing domain, binds siRNAs
RBM1CTR	C-terminal region found in hnRNPs
PAM2	PABP-interacting motif PAM2, found in RBPs
Xpo1	exportin 1 domain, RNA transport or RBP transport proteins
S1	S1 ssRNA-binding domain
HGTP_anticonodon	Anticodon binding domain, found in aminoacyl-tRNA synthetases
tRNA-synt_2b	tRNA synthetase class II core domain (G, H, P, S and T), core catalytic domain of tRNA synthetases
Piwi	Piwi domain (P-element induced wimpy testis), posttranscriptional silencing domain, dsRNA guide hydrolysis of ssRNA
CSD	cold-shock domain, ssRNA/ssDNA binding
Ribosomal_L7Ae	domain found in ribosomal proteins L7Ae/L30e/S12e/Gadd45
RNase_Zc3h12a	ssRNA endonuclease domain found in Zc3h12a proteins, member of the NYN domain family
Anticodon_1	tRNA anticodon-binding domain, found in tRNA synthetases
R3H	R3H domain, R-x3-H conserved core, binds ssRNA/ssDNA

Table 1. RNA-binding domains. List of the RBDs commonly found in RBPs by Gerstberger *et al.* (2014) Nat Rev Genet. 15(12):829-45.

RBPs can also be classified based on their RNA targets (Fig. 1). This type of classification also suggests the post-transcriptional pathways in which they are involved.

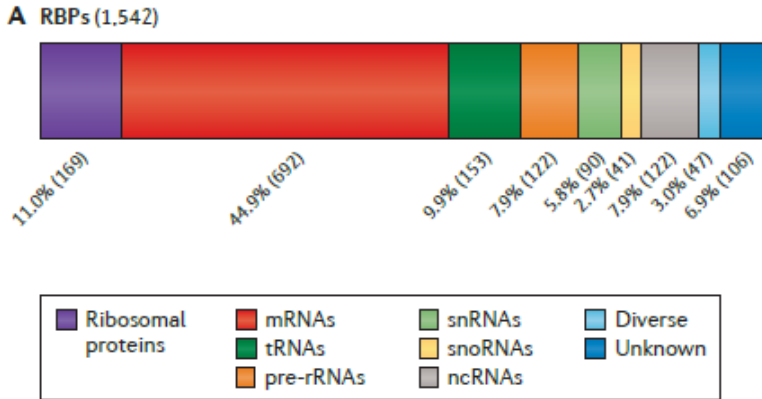


Figure 1. RBPs classification. RBP families can be grouped according to their respective targets: ribosomal proteins, mRNA, tRNA, pre-ribosomal RNA, small nuclear RNA (snRNA), small nucleolar RNA (snoRNA), non-coding RNA (ncRNA); diverse targets and unknown targets (*Gerstberger et al (2014) Nat Rev Genet. 15(12):829-45*).

The diversity of RBDs suggests that many RBPs remain yet to be identified. Accordingly, hundreds of novel non-canonical RBPs have been recently identified. These newly identified proteins are involved in diverse biological processes and belong to different protein families. Interestingly, many of them exhibit enzymatic activities, thus suggesting the existence of a crosstalk between RNA biology and other fundamental cell processes such as metabolism⁴. Unexpectedly, hundreds of the newly identified RBPs do not contain any of the classical RBDs, but they rather bind to RNA through the presence of intrinsically disordered protein regions in their sequence, that can be grouped into RS-rich, RG-rich, and other basic sequences, which mediate both specific and non-specific interactions with RNA⁵. These recent results confirm that the repertoire of RBPs is much higher than expected, probably because eukaryotes evolved highly specific post-transcriptional processes to fine-tune gene expression, a process in which RBPs act as main players⁶.

1.2. RNA-binding proteins and the re-programming of gene expression in cancer

Each cellular process – such as proliferation, differentiation, development, apoptosis, senescence, carcinogenesis - relies on the re-programming of gene expression⁷. Although initially described as a “simple” three-step process, by which the flow of genetic information proceeds from DNA, passing through RNA, to the final protein, it is now clear that gene expression is more complex than expected and can be regulated at multiple levels⁸. Post-transcriptional control, that influence mRNA metabolism and translation once it is transcribed, represents one of the most intricate layer of gene regulation; therefore, it is not surprising that in pathologies such as cancer, cells strongly rely on this mechanism to adapt to the microenvironmental changes to support tumour growth and progression⁹.

This complex layer of regulation involves spatially and temporally separated - but deeply integrated - mechanisms, that requires the intervention of hundreds of players¹⁰.

RNA-binding proteins are considered one of the key players of post-transcriptional control (Fig. 2). Through the interaction with other proteins and coding/non-coding RNAs, they form ribonucleoprotein complexes that are involved in every aspect of RNA biology, from pre-mRNA splicing and polyadenylation to RNA modification, transport, localization, and translation¹¹. Given their pivotal role in post-transcriptional events, it is common that alterations of RBPs can lead to several diseases such as muscular atrophies, neuropathies and cancer¹².

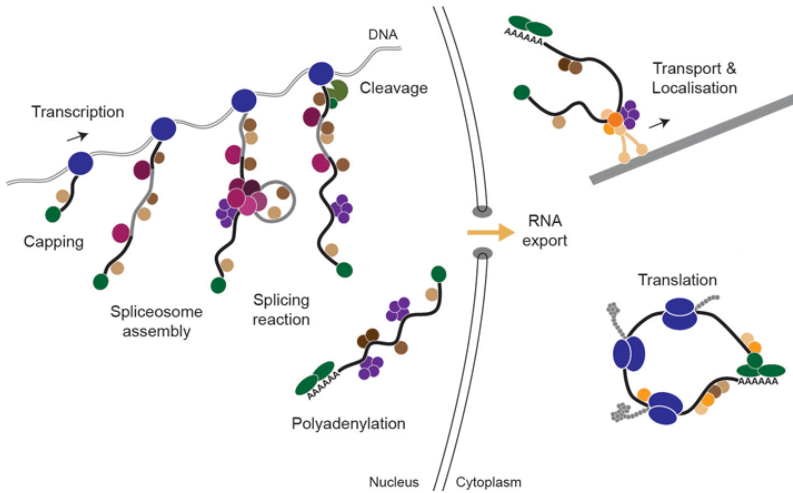


Figure 2. Overview of the complex life of a eukaryotic mRNA. A plethora of RNA-binding proteins guides the mRNA through multiple nuclear and cytoplasmic processing steps, which ultimately determine its fate and function in the cell (https://www.bmls.de/Computational_RNA_Biology/aboutus.html).

Accordingly, several and recent studies provided strong evidence that RBPs can be abnormally expressed in cancer in comparison to adjacent normal tissues, and this expression directly correlates with patient prognosis^{13,14,15}.

For example, the RBP SAM68 is upregulated in breast cancer and its knockdown inhibits cell proliferation through the upregulation of the cell-cycle inhibitors p21 and CDKN1B/p27, at both mRNA and protein levels¹⁶. In a similar way, the transcription factor E2F1 increases the mRNA levels of the RBP Quaking (QKI) which, in turn, negatively regulates E2F1 activity, delaying S-phase entry by increasing the stability of p27 mRNA and decreasing FOS mRNA expression in colon cancer¹⁷. Another well-studied family of RBPs overexpressed upon malignant transformation, are the insulin-like growth factor 2 mRNA binding proteins (IGF2BP). The expression of these proteins often correlates with poor prognosis. Overexpression of IGF2BP increases Myc and KRAS expression in colorectal cancer cell lines with increased proliferation, and its intestine deletion in a mouse model of intestinal tumorigenesis is responsible for the reduction of

tumour number^{18,19}. The RBP Hu-antigen R (HuR) either promotes the translation or enhances the stability of several mRNA targets encoding pro-survival proteins such as BCL2, MCL1, PTMA, and SIRT1 in several malignancies²⁰. In line with these results, deletion of HuR in a transgenic murine model leads to apoptosis of progenitor cell populations of intestinal systems²¹. In a similar way, La ribonucleoprotein domain family member 3 (LARP3) binds and enhances the translation of BCL2, MDM2, and XIAP mRNAs, promoting cell survival in myeloid leukemia²². Moreover, RBPs mediate different post-transcriptional events related to motility and invasiveness that are altered in cancer cells. An example is the RBP upstream of N-Ras (UNR), which was very recently demonstrated to be overexpressed in melanomas where it promotes invasion and metastasis through a translational regulation of its pro-metastatic target mRNAs, VIM and RAC1¹⁵.

Taken together, these studies provide strong evidence that the post-transcriptional regulation of gene expression exerted by RBPs might be involved in every process leading to tumour development - usually through the dysregulation of their mRNA targets - from the evasion of cell death to the deregulated proliferation and invasion.

1.3. Dysregulated RBP-dependent post-transcriptional mechanisms in cancer

As mentioned above, RBPs act as main regulators of each step of the mRNAs life cycle, including alternative splicing, polyadenylation, stability, subcellular localization, and translation. Therefore, their roles in cancer can be attributed to the functional dysregulation of one or more of these post-transcriptional mechanisms.

1.3.1. Alternative splicing

Alternative splicing is a commonly altered mechanism in cancer, therefore the interest in targeting the spliceosome machinery has grown over the years²³. The most frequent alterations of this process are attributable to altered RBPs function. Among the well-known and characterized RBPs families involved in alternative splicing there are the heterogeneous ribonucleoproteins particles (hnRNPs) and the serine/arginine-rich (SR) proteins²⁴. Both proteins are dysregulated in a wide range of cancers^{25,26,27}. Another splicing factor frequently downregulated in lung cancer, which is associated with poor

prognosis, is the QKI. QKI is a signal transduction and activators of RNA (STAR) family member that, in normal cells, selectively represses the inclusion of exon 12 in the NUMB mRNA by a competitive mechanism with the splicing factor SF1, thus promoting the expression of a NUMB mRNA isoform that inhibits proliferation by negatively regulating the Notch pathway (Fig. 3)²⁸. In a similar way, the splicing factor RBM10 is downregulated in lung adenocarcinomas, where it promotes NUMB mRNA exon 9 skipping, leading to the expression of a NUMB isoform that blocks proliferation by inhibiting Notch pathway²⁹.

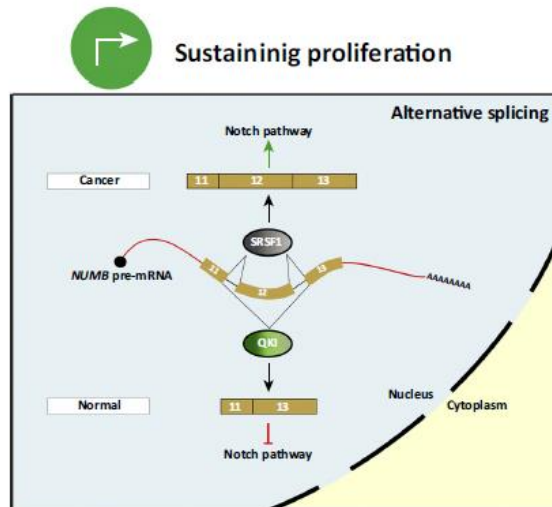


Figure 3. Alternative splicing. In cancer cells, QKI is frequently downregulated, leading to the expression of a NUMB mRNA isoform with exon 12 encoding a protein which is able to activate the Notch pathway and cell proliferation (*Pereira B et al (2017) Trends Cancer. 3(7):506-528*).

1.3.2. Alternative polyadenylation

Another crucial step in the processing of eukaryotic pre-mRNAs, is the addition of a poly(A) tail to their 3'-end by the endonucleolytic cleavage of the transcript and the addition of a stretch of adenosines. This step is required for nuclear export and to ensure mRNAs stability and efficient translation³⁰. RBPs are also in charge for this process. As for the alternative splicing, alternative polyadenylation (APA) is responsible for the existence of multiple transcripts from a single

gene. APA can alter either the coding sequence - affecting the function of the protein product - or the 3' untranslated region (3'-UTR) - affecting the stability, localization, and translation of the target mRNA³¹. Hence, it is not surprising to find that APA is frequently altered in cancer. Cancer cells mostly express mRNA isoforms with shorter 3'-UTR, with consequent loss of 3'-UTR repressive elements that leads to the production of tenfold more protein in transformed cells by APA³². A well characterized family of RBPs involved in this process is the cytoplasmic polyadenylation element binding (CPEB) proteins family. For example, CPEB4 is overexpressed in melanomas, glioblastomas, and pancreatic ductal adenocarcinomas^{33,34}. In melanomas, this protein controls polyadenylation and increased translation of MITF and RAB72A, targets involved in the G1/S transition, thereby promoting proliferation³⁴. In pancreatic cancer, CPEB4 controls poly(A) tail elongation and abnormal translational activation of mRNAs that are silenced in normal tissue, including the mRNA of tissue plasminogen activator (TPA), thus promoting tumour growth, invasion, and vascularization (Fig. 4)³³. Recently, it has been demonstrated a coordinated and sequential post-transcriptional role in the regulation of vascular endothelial growth factor (VEGF), by CPEB4 and CPEB1. Briefly, in transformed endothelioma cells, CPEB1 generates shorter 3'-UTR isoforms of both CPEB4 and VEGF mRNAs, thus excluding translation inhibitory elements. This trigger CPEB4 transcript stabilization and protein expression which, in turn, increases the translation of VEGF mRNA by enhancing its cytoplasmic polyadenylation³⁵.

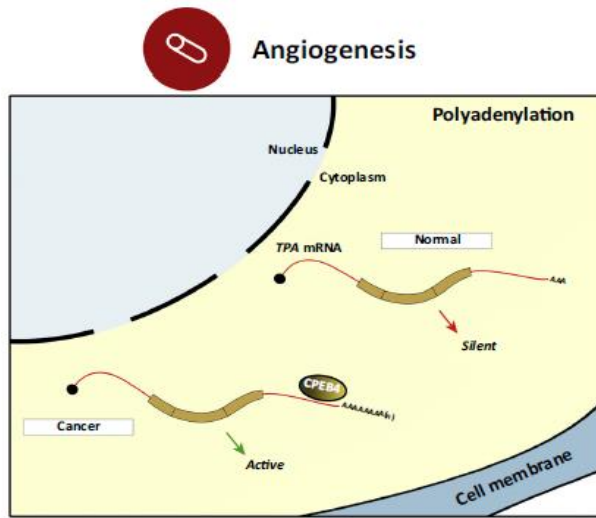


Figure 4. Alternative polyadenylation. CPEB4 is overexpressed in cancer cells and induces poly(A) tail elongation and translational activation of the TPA transcript, which supports tumour growth, invasion, and vascularization (*Pereira B et al (2017) Trends Cancer. 3(7):506-528*).

1.3.3. mRNA Stability

In addition to the poly(A) tail, eukaryotic mRNAs also incorporate a 5'-cap structure during their biogenesis. This structure represents another element responsible for the regulation of mRNAs along with the 3'-poly(A). mRNAs must be cleaved by an endonuclease recognizing specific cis-destabilizing elements of one of these structures to drive them towards decay³⁶. Among these elements, the most frequent in the 3'-UTR is the AU-rich element (ARE), which is found in about 16% of all transcripts³⁷. In response to several stimuli, ARE elements are recognized by a family of RBPs known as ARE-binding proteins (AUBPs). AUBPs are required for both the destabilization or the stabilization of the target transcript according to the cellular signals.

The importance of mRNA stability and AUBPs in cancer is pointed out by the evidence that oncogenes, growth factors, cell-cycle genes, and inflammatory mediators are over-represented among the ARE-containing transcripts³⁸. For example, HuR is overexpressed in multiple cancer types, where it is responsible for increased proliferation by enhancing the stability of several ARE-containing

mRNAs. Among them, cyclins A1 and B in colorectal cancer, cyclin D1 in human cervical carcinoma, and cyclin E1 in breast cancer^{39,40,41}(Fig. 4). Another example is the RNA-binding protein hnRNP D or AUF1, which has either stabilizing or destabilizing effects in different systems. A specific isoform of this protein, named p37AUF1, is able to induce spontaneous sarcomas when overexpressed in mice and leads to the accumulation of cancer-associated transcripts like Myc, Fos and cyclin D1⁴². Conversely, AUF1 can also act as a tumour suppressor by destabilizing mRNAs encoding the anti-apoptotic protein BCL2 and the proinflammatory factors GM-CSF, IL-6, IL-10, and TNF- α ⁴³.

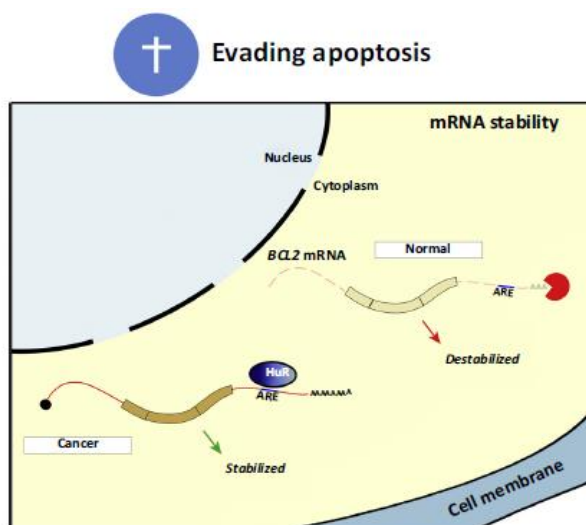


Figure 4. mRNA stability. HuR stabilization of the anti-apoptotic BCL2 ARE-containing mRNA, eliciting survival. In its absence, the transcript is destabilized and targeted for decay by a process involving poly(A) tail shortening (Pereira B et al (2017) Trends Cancer. 3(7):506-528).

1.3.4. mRNA localization and translation

RBPs also play a major role in the intracellular localization - and consequent translation - of mRNAs by binding to sequences located in their 3'-UTR. Usually, RBPs associate with mRNAs and form multi-complexes that link transcripts to cytoskeletal molecular motors, which, in turn, send RNPs to specific subcellular addresses⁴⁴. Transporting mRNAs rather than proteins is significantly

advantageous for a cell for several reasons: i) a single mRNA can be translated in more protein molecules, so the transport of one mRNA instead of multiple proteins is cost-effective; ii) the mRNAs transport prevents proteins from carrying out their functions before reaching their final destination, where such function is actually required; iii) localized translation facilitates the incorporation of proteins into macromolecular complexes by generating high local protein concentrations of different subunits, as demonstrated for the seven members of the Arp2/3 complex, whose localized synthesis increases the chances of subunits finding each other for assembly⁴⁵. These processes allow the fine-tuning of gene expression in both space and time; therefore, it is not surprising that this mechanism is frequently altered in cancer.

Almost all the major oncogenic signalling pathways altered in cancer, such as PI3K/AKT/mTOR, RAS/MAPK, and Wnt/b-catenin lead to dysregulation of translation. The presence of sequence-specific regulatory elements in the mRNAs guides the preferential translation of the oncogenic program in cancer⁴⁶. Among them, the internal ribosome entry site (IRES) - a structural element that can be found either in the 5'-UTR or the coding region, thereby triggering to the synthesis of different isoforms - is one of the most studied. IRES elements promote translation by recruiting the ribosome through an association with IRES trans-acting factors when cap-dependent translation is inhibited, a condition frequently found in tumours⁴⁷.

Several RBPs play a major role in both the transport and translation of their target mRNAs. For example, LARP3 interacts with the laminin B1 IRES, positively modulating its translation, which in turn enhances the epithelial to mesenchymal transition (EMT) programme that promotes the survival and invasiveness of hepatocellular cancer cells⁴⁸.

One of the best example of an RBP involved in both the transport and localized translation of its targets is provided by the insulin-like growth factor 2 mRNA binding protein 1 (IMP1), a member of the conserved VICKZ family of RBPs. IMP1 binds to actin B (ACTB) transcript in the nucleus and inhibits its translation in the cytoplasm (Fig. 5). ACTB translation occurs only when the RBP/mRNA complex reach the periphery of the cell, following the phosphorylation of IMP1 by Src-kinase - whose activity is spatially restricted - in a specific residue responsible for the RNA binding⁴⁹. IMP1 is highly

expressed in primary tumour tissues like breast, colon and lung carcinomas, whereas it is downregulated in metastatic cells, and this downregulation impairs the transport and localized translation not only of ACTB but of other motility-related target mRNAs, such as α -actinin, E-cadherin and the Arp2/3 complex, thus promoting cell migration and metastatic cells growth^{50,51}.

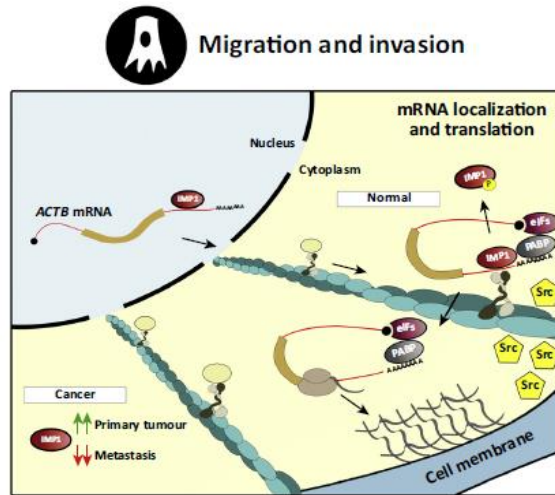


Figure 5. mRNA subcellular localization and translation: IMP1 protein recognizes specific sequences in the 3'-UTR of the β -actin mRNA, controlling its transport and subsequent translation in polarized cells. In cancer, dysregulation of this process impacts on the turnover of focal adhesions and protrusion dynamics, and this plays an important role in generating cells with a more motile and invasive behaviour (Pereira B et al (2017) Trends Cancer. 3(7):506-528).

All these control events may act independently from one another or be coordinated in space and time, allowing RBPs to fine-tune gene expression in cancer. In some cases, a single RBP is involved in the regulation of a set of targets within specific post-transcriptional layers, while, in others, the combinations of two RBPs regulate a single transcript, which, depending on synergistic and/or antagonistic interplay, can yield different outcomes⁵².

1.4. Translational control in cancer: role of the ribosome-bound chaperones

Among the aforementioned mechanisms, mRNA translation represents one of the main dysregulated processes in cancer cells, which exhibit an increase of protein synthesis and selective translation of specific mRNAs that promote tumour cell survival, sustained proliferation, invasion and metastasis. Accordingly, the overexpression of several components of translation initiation machinery, such as eIF2 α , eIF3a, b, c, h, eIF4A, eIF4G1 and eIF5A was shown to cause or to strongly correlate with different cancer types, such as melanoma, cervix, breast, testis, prostate, hepatocellular, squamous cell lung and ovarian cancer⁵³. Together with initiation factors, the overexpression of elongation factor has been associated to cancer as well. For example, the overexpression of EF1A1 and EF1A2 has been found in ovarian and breast cancer⁵⁴.

In eukaryotes, translation consist of three steps: initiation, elongation and termination. Accumulation of errors - usually associated to genetic instability - during each of these steps, can be responsible for the onset and progression of malignancies through the increased production of damaged and/or misfolded proteins, which is now considered a hallmark of cancer cells⁵⁵. There are several sources that can generate defective translation products, therefore the ability of cells to detect and remove errors before the polypeptide is fully functional represents an opportunity to avoid their accumulation. To ensure the correct synthesis and folding of newly synthesized proteins, eukaryotic cells evolved protein quality control machineries, which also play crucial roles in cancer cells⁵⁶. In this context, molecular chaperones are key players, being involved in the folding of both newly translated and stress-denatured proteins. While these processes are equivalent in prokaryotes - with chaperones associating equally to substrates generated either by synthesis or stress-denaturation - eukaryotes evolved a distinct and elaborate machinery of ribosome-bound chaperones that interacts with and facilitates folding of nascent polypeptides in a co-translational manner⁵⁷. *Albanese et al* demonstrated the existence of this machinery in yeast and referred to these translation-associated chaperones as Chaperones Linked to Protein synthesis (CLIPs). In line with this evidence, the so called “mammalian ribosome-associated complex” has been identified in higher eukaryotes. This complex comprises dynamically interacting

factors, including HSP70, serving multiple functions, such as co-translational sorting, folding, and covalent modification of newly synthesized polypeptides⁵⁸.

When cell fails to remove errors, damaged proteins are targeted to degradation, mainly by the ubiquitin-proteasome system (UPS), which represents one of the major component of the quality control machinery, that can mark proteins for destruction while they are being synthesized⁵⁹. Thus, both the chaperone network and the ubiquitination system are fundamental for the quality control of newly synthesized proteins, to ensure a correct folding and an efficient clearance of translation-defective products. By exerting a translational control on tumour-promoting/suppressing proteins, this complex protein quality control machinery is crucial for cancer development and progression.

1.5. The molecular chaperone TRAP1

TRAP1 (Tumour Necrosis Factor receptor-associated protein 1) is a molecular chaperone belonging to the heat shock protein 90 (HSP90) chaperone family - therefore also known as HSP75 - with whom it shares 26% identity and 45% similarity⁶⁰. Despite their similarity, TRAP1 and HSP90 do not share the same functions and show distinct features. While HSP90 exerts its function mainly in the cytoplasm, TRAP1 is mostly localized in mitochondria, where it contributes to protection from apoptosis induced by several stresses⁶¹.

TRAP1 was discovered almost at the same time by two different groups: on one hand, it was identified as a type I tumour necrosis factor receptor-associated protein by a yeast-based two hybrid screening⁶², on the other hand it was characterized as a chaperone of the retinoblastoma protein⁶³.

TRAP1 is a protein that plays a controversial role in tumour biology. In fact, it was found strongly expressed in tumour cells of adenocarcinomas of pancreas, breast, colon, and lung, whereas normal matched epithelia contain very low levels of this chaperone⁶⁴. Accordingly, this protein was found overexpressed in 17/26 human colorectal carcinomas⁶⁵. It was also found abundantly and ubiquitously expressed in human high-grade prostatic intraepithelial neoplasia, Gleason grades 3 prostatic adenocarcinomas, and metastatic prostate cancer, but largely undetectable in normal prostate or benign prostatic hyperplasia *in vivo*⁶⁶. Conversely, recent data show a more

complex scenario with a lower expression of TRAP1 in lung cancer and cisplatin-resistant ovarian cancer cells^{67,68,69}.

Although TRAP1 has been considered for long a mitochondrial protein and its functions investigated on the basis of this localization, it was firstly identified as non-mitochondrial^{62,63}. Accordingly, extra-mitochondrial localization of TRAP1 have been shown by electron microscopy⁷⁰. Moreover, *Ghosh et al.* found it as a component of the membrane proteome⁷¹.

1.5.1. TRAP1 outside the mitochondria: coupling of protein synthesis and degradation

In line with such evidence, in 2012, our group performed a Liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis, in which many cytoplasmic proteins were reported as putative TRAP1 partners. Consistently, TRAP1 was found on the outer side of the endoplasmic reticulum (ER), opening a new scenario on the functions of this protein in cancer cells⁷².

Our studies demonstrated that the endoplasmic reticulum TRAP1 interacts with TBP7/Rpt3, an ATPase protein of the proteasome regulatory subunit. This interaction is involved in the co-translational protein quality control of nuclear encoded-mitochondrial proteins; in particular, the calcium binding protein Sorcin isoform B and F1ATPase β subunit. Both these proteins show lower expression and higher ubiquitination levels upon TRAP1 silencing in human colorectal cancer cells HCT116. Moreover, TRAP1 and/or TBP7 interference increases total amount of ubiquitinated proteins. This phenotype can be attributed only to the extramitochondrial fraction of TRAP1, since the transfection of a TRAP1 mutant lacking the N-terminal mitochondria targeting sequence – therefore unable to enter mitochondria – is able to rescue this phenotype⁷². The co-translational basis of this mechanism was suggested by the evidence that TRAP1 associates with ribosomes and translational factors such as eIF4A, eEF1A and eEF1G. Consistently, few years ago, *Pandolfi PP et al.* identified a set of riboproteome components in mammalian cells through a SILAC-based mass spectrometry approach, in which they found TRAP1 as a component of the riboproteome⁷³. Furthermore, our group demonstrated that in presence of TRAP1 there are enhanced phosphorylation levels of the translation factor eIF2 α , both in basal or stress conditions, thus leading to the attenuation of cap-dependent

translation in favour of the IRES-dependent one. This mechanism prevents the accumulation of damaged or misfolded proteins and facilitates the synthesis of selective cancer-related proteins⁷⁴. Accordingly, TRAP1 silencing sensitizes cells to apoptosis induced by novel antitumoral drugs that inhibit cap-dependent translation, such as Ribavirin or 4EGI-1, and reduces the ability of cells to migrate through the pores of transwell filters in the presence of these drugs⁷⁵. Finally, TRAP1-dependent regulation of protein synthesis is involved in the migratory behaviour of different cancer cells by regulating p70S6 kinase expression and activity, and EMT associated genes^{76,77}.

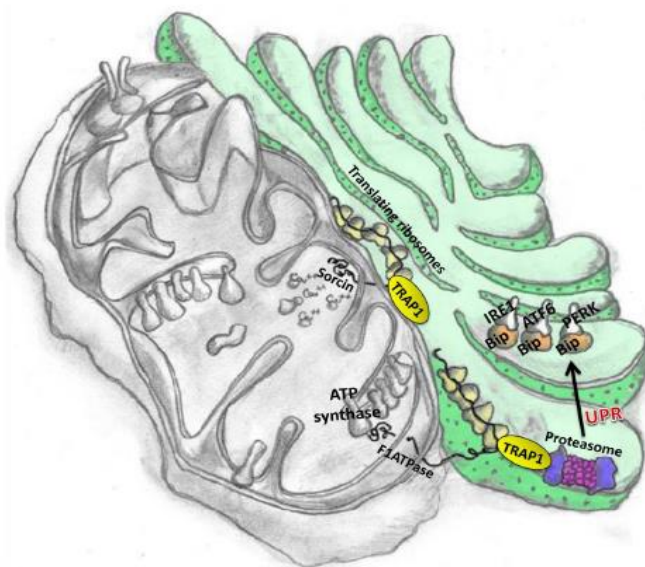


Figure 6. TRAP1 outside the mitochondria. Overview of TRAP1 regulation of protein synthesis (Matassa DS et al (2017) Encyclopedia of Signaling Molecules, 2nd Edition. ISBN: 978-3-319-67198-7).

1.5.2. TRAP1 in mitochondria

1.5.2.1. TRAP1 as an antiapoptotic protein

According to its prevalent mitochondrial distribution and the great research interest in the characterization of TRAP1 mitochondrial functions, the first role assigned to this chaperone was the protection against mitochondrial apoptosis. In the mitochondria of tumour cells, TRAP1 is involved in a chaperone network that implies the formation

of a ternary complex - together with HSP90 and the immunophilin cyclophilin D - that regulates the permeability transition pore opening, maintaining mitochondrial homeostasis, and antagonizing the pro-apoptotic function of cyclophilin D in permeability transition⁷⁸. The mitochondrial TRAP1 also forms a cytoprotective complex with the mitochondrial isoform of the calcium-binding, antiapoptotic protein Sorcin in colorectal cancer cells⁷⁹. Several observations suggest that oxidative stress prevention may be likely involved in (and part of) TRAP1 regulation of cell death. Accordingly, cells expressing high levels of TRAP1 show increased levels of the scavenging tripeptide GSH and are more resistant to oxidative stress, also showing cross-resistance to chemotherapeutics⁶⁵. Furthermore, TRAP1 prevents oxidative-stress-induced apoptosis in neurons as a downstream effector of PINK1, and the dysregulation of this mitochondrial pathway seems to be involved in the pathogenesis of Parkinson's Disease (Fig. 7). In line with this evidence, TRAP1 overexpression causes a decrease of cleaved Caspase 3 and PARP, commonly considered as apoptotic markers. TRAP1 interference, as well as the use of dominant negative mutants of TRAP1, sensitized oxidative stress/chemoresistant cells to cell death inducers, thus providing the evidence that TRAP1 is an important player in the development and the maintenance of these phenotypes⁷⁹.

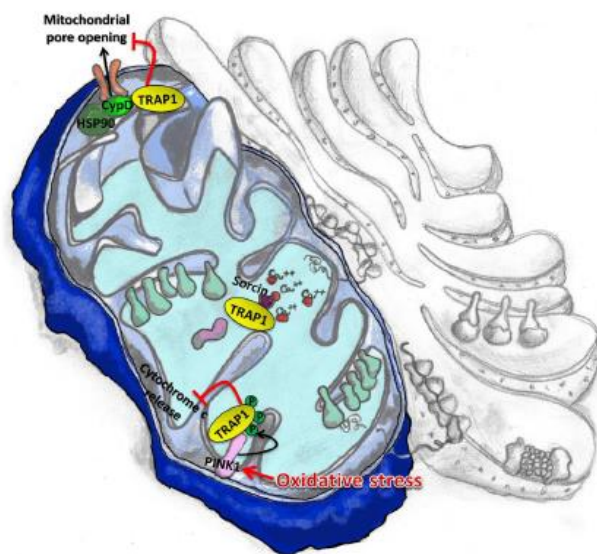


Figure 7. TRAP1 in mitochondria. Overview of the antiapoptotic mechanisms in which TRAP1 is involved (*Matassa DS et al* (2017) Encyclopedia of Signaling Molecules, 2nd Edition. ISBN: 978-3-319-67198-7).

1.5.2.2. TRAP1 as a modulator of cell metabolism

In the last few years, TRAP1 has also emerged as a critical regulator of mitochondrial respiration through the direct binding to respiratory complexes⁸⁰. Data showed that TRAP1 interact with complex II and IV of the electron transport chain and inhibits Succinate dehydrogenase (SDH) activity, without affecting complex II protein levels or mitochondrial mass, thus contributing to the Warburg phenotype⁸¹. As a result, TRAP1 yields a reduced oxygen consumption rate (i.e. reduced mitochondrial respiration) in different cell lines, thus inducing a metabolic shift toward glycolysis and a “Warburg phenotype”, and decreased fatty acid oxidation⁸⁰. Conversely, another group found an interaction between TRAP1 and the tyrosine-protein kinase c-Src, which is known to stimulate complex IV activity and to enhance oxidative phosphorylation, suggesting that the impact of TRAP1 on mitochondrial respiration could be mediated by c-Src (Fig. 8)⁸².

We have recently reported that TRAP1 reduces oxidative phosphorylation rate in ovarian cancer cells, but in such system oxidative phosphorylation favours drug resistance, thus providing to TRAP1 oncosuppressive properties in this specific context⁶⁹. Taken together, these studies revealed that the regulation of cancer cell metabolism by TRAP1 seems to have contextual effects on cancer onset and progression, thus favouring the oncogenic phenotype in glycolytic tumours⁸¹, while being negatively selected in tumours mostly relying on oxidative metabolism⁶⁹.

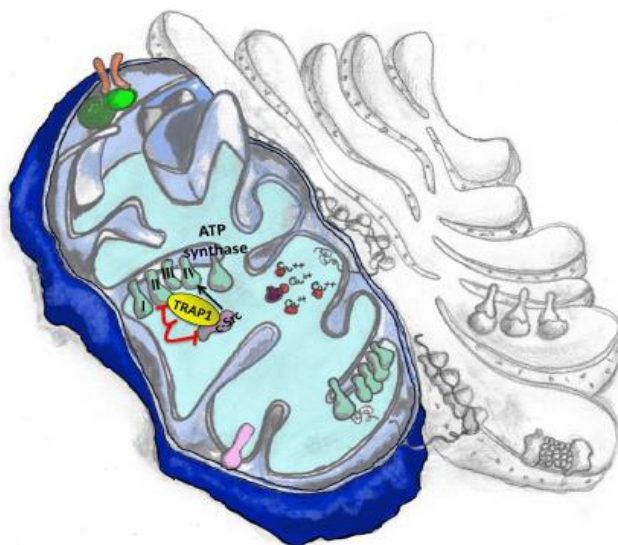


Figure 8. TRAP1 in mitochondria. Overview of cell metabolism modulation by TRAP1 (Matassa DS et al (2017) Encyclopedia of Signaling Molecules, 2nd Edition. ISBN: 978-3-319-67198-7).

2. AIM

TRAP1 is a molecular chaperone involved in the quality control of mitochondria-destined proteins, through the regulation of their co-translational ubiquitination and degradation. However, mechanisms of substrate recognition are still unknown. The question that gave rise to the present study is whether TRAP1 identify and binds some interacting partners through its interaction with RNA. Several lines of evidence suggest that TRAP1 might directly recognize and bind its substrates as mRNAs, being potentially involved in their transport to mitochondria and localized translation. In fact, some unpublished data by our group strongly supports this hypothesis, with an increase of actively translating ribosomes in the proximity of mitochondria following TRAP1 overexpression, as demonstrated by increased number of proximity ligation foci between the mitochondrial protein import channel Tom20 and active ribosomal protein phospho-rpS6 (Fig. 9).

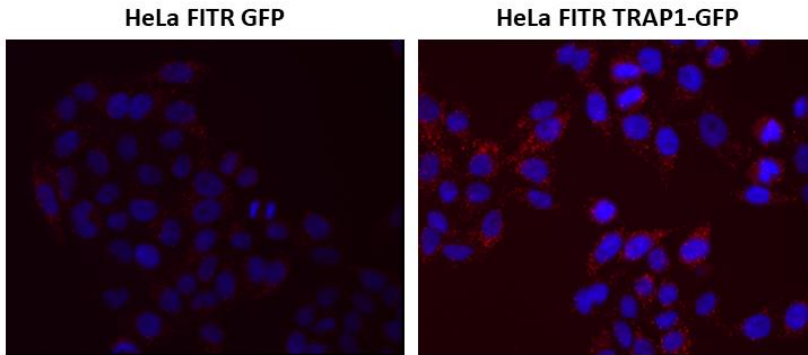


Figure 9. TRAP1 overexpression increases protein synthesis in the vicinity of mitochondria. 24 hours after induction, HeLa eGFP and TRAP1-eGFP were fixed, permeabilized and hybridized with anti-TOM20 and anti-phospho rpS6 antibodies and subjected to proximity ligation assay (PLA). PLA allows visualization of distinct fluorescent spots (red points) when the two target epitopes are distant no longer than 40 nm. Nuclei were stained with DAPI (blue).

Moreover, it has been previously demonstrated that TRAP1 binds the 3'-UTR of the mitochondrial ribosomal protein S12⁸³. Finally, TRAP1 was identified as a putative RBP in HeLa cells, following the

interactome capture technique developed by Castello A. and colleagues⁴.

TRAP1 substrates recognition mechanisms could also involve other players. Accordingly, preliminary analyses by mass spectrometry revealed that some TRAP1 interactors are validated or putative RBPs. Among those, we focused our attention on Protein Syndesmos (SDOS)/Nudt16l1 a paralog of the nuclear Nudt16, but lacking the catalytic activity due to critical sequence changes within the catalytic NUDIX domain and therefore unable to perform its canonical mRNA decapping activity⁸⁴. Although initially described for its role in the assembly of focal adhesions and actin stress fibers - through the interaction with Syndecan4, Paxillin and its homolog Hic-5⁸⁵ - a very recent and interesting research demonstrated that SDOS plays a role in DNA damage response. In fact, it interacts with the Tudor domain of the P53-binding protein 1 (53BP1) influencing its function during double-strand break repair by masking its demethylated lysine 20 of histone H4 binding motif⁸⁶. Interestingly, SDOS as well was found in the list of the HeLa RBPs repertoire identified by interactome capture, strongly supporting the hypothesis that SDOS and TRAP1 might have RNA-binding properties.

Starting from these observations, and more specifically, the aim of my study is to:

- Characterize TRAP1/SDOS interaction;
- Analyse SDOS localization and functions in cancer cells;
- Demonstrate that TRAP1 and/or SDOS are RBPs;
- Identify TRAP1 and SDOS-directly regulated targets;
- Identify and characterize relevant pathways affected by TRAP1 and SDOS, based on the identified substrates.

3. MATERIALS AND METHODS

3.1. Cell culture

Human HCT116 colon carcinoma cells and human cervical carcinoma HeLa cells were purchased from American Type Culture Collection (ATCC) and cultured in McCoy's 5A medium (HCT) and DMEM (HeLa). Both culturing mediums contain 10% fetal bovine serum, 1.5 mmol/L glutamine. The authenticity of the cell lines was verified at the beginning of the project by STR profiling, in accordance with ATCC product description. HeLa Flp In TRex (FTR) cell line were kindly provided by Dr. Matthias Gromeier (Duke University Medical Center, Durham, USA). Generation of the HeLa Flp In TRex stable cell lines expressing the eGFP-fusion proteins or the short hairpin RNA, was performed as described in the manufacturer's protocol (Flp In TRex, Invitrogen). HeLa Flp In TRex cells were cultured in DMEM supplemented with 10% fetal bovine serum, 1.5 mmol/L glutamine, and appropriate selective antibiotics. Addition of tetracycline induces proteins as described in⁴.

3.2. Plasmid generation and transfection procedures

Full-length SDOS-myc cloned into pcDNA 3.1 myc-his vector was obtained as previously described⁷². MOV10-YFP and eGFP alone cloned into pcDNA5/FRT/TO (Invitrogen) were kindly provided by Prof. Matthias Hentze, EMBL/Heidelberg Univ. "Molecular Medicine Partnership Unit". For TRAP1-eGFP and SDOS-eGFP plasmids generation, HeLa cDNA library and eGFP plasmid were used as templates for fusion PCR. Resulting chimeric cDNAs were cloned into pCDNA5/FRT/TO. TRAP1-Flag-HA and SDOS-Flag-HA plasmids were obtained in the same way by using the Flag-HA tagged vector as template kindly provided by Dr. Alfredo Castello, Department of Biochemistry, University of Oxford. Transient transfection of DNA plasmids was performed with the Polyfect Transfection Reagent (Qiagen - 301105) according to the manufacturer's protocol. TRAP1 and SDOS transient silencing were performed with siRNAs purchased from Qiagen (TRAP1: cat. no. SI00115150; SDOS: cat. no. SI00713293). For control experiments, cells were transfected with a similar amount of scrambled siRNA (Qiagen; cat. no. SI03650318). Transient transfections of siRNAs

were performed using HiPerFect Transfection Reagent (Qiagen - 301704) according to the manufacturer's protocol.

3.3. Western blot and Immunoprecipitation analysis

Equal amounts of protein from cell lysates was subjected to SDS-PAGE and transferred to a PVDF membrane (Millipore). WB analysis were performed as described in⁸⁷. Protein immunoprecipitations were carried out as previously described⁷⁴. eGFP-fusion proteins were immunoprecipitated with GFP_trap agarose beads (GFP_trapA: Chromotek) according to manufacturer's instructions. Where indicated, protein levels were quantified by densitometric analysis using the software ImageJ. The following antibodies were used for WB, immunofluorescence and immunoprecipitation: anti-TRAP1 (sc-13557), anti- β -ACTIN (sc-69879), anti-GAPDH (sc-69778), anti-Bip (sc-1051), anti VDAC1 (sc-8828), anti-F1ATPase (sc-16690), anti-eGFP (sc-81045), anti-MYC (sc-40), anti-Vinculin (sc-73614), anti-PARP1 (sc-25780); anti-SDOS (HPA044186), anti-FLAG (FT425), anti-TMEM107 (HPA052555) from Sigma-Aldrich; anti-PABP1 (GTX113954) from Genetex; anti-rpL3 was kindly provided by Prof. Giulia Russo, Department of Pharmacy, University of Napoli "Federico II".

3.4. RNA extraction and qPCR analysis

RNA extraction procedures and qPCR analysis were performed as described in⁷².

3.5. ³⁵S Met/³⁵S Cys labelling

HeLa FITR and HCT116 cells were seeded in a 6-well plate. HeLa eGFP and HeLa SDOS-eGFP cells were induced for 24 hrs while HeLa sh-eGFP and sh-SDOS for 48 hrs with 1 μ g/mL doxycycline. HCT116 were transfected with a SDOS-directed siRNA. For control experiments, cells were transfected with a similar amount of non-targeting control siRNA. Following proteins induction or silencing, cells were incubated in cysteine/methionine-free medium (Sigma-Aldrich) for 15 min followed by incubation in cysteine/methionine-free medium containing 50 μ Ci/ml ³⁵S-labeled cysteine/methionine (Perkin-Elmer) for 30 min. Cells were then washed with PBS and lysed. Ten μ g of total protein extract was analysed by SDS-PAGE and autoradiography.

3.6. Confocal microscopy and Fluorescence in situ hybridization analysis (FISH)

HeLa SDOS-eGFP cells were seeded on coverslips and prepared for immunofluorescence analysis as previously described⁷⁶ following Sodium Arsenite treatment. For FISH analysis, cells were directly grown onto coverslips. Following Sodium Arsenite treatment, cells were washed once with PBS and fixed in 4% paraformaldehyde for 10 minutes at room temperature. Cells were then permeabilized by treatment with 100% Ethanol for 10 minutes, rehydrated with 70% Ethanol and equilibrated in TRIS-HCl pH 8 for 10 minutes. Hybridization was performed 2 hrs at 37°C in 30 µl of a mixture containing 10% dextran sulphate, 2 mM vanadyl-ribonucleoside complex, 1% RNase-free BSA, 0,5 µg/µl of E. coli tRNA, 2X SSC, 20% formamide, 2 ng/µl of Alexa-594 oligo-dT probe. Cells were then washed once with SSC 4X for 10 minutes, twice with SSC 2X for ten minutes, and once with SSC 2X containing 0,1% Triton for 15 minutes followed by two more washes with SSC 2X. Coverslips were mounted with appropriate mounting medium and signal was detected by confocal microscopy analysis.

3.7. Duolink in situ proximity ligation assay

Duolink in situ proximity ligation assay (Sigma-Aldrich - DUO92101) was performed according to the manufacturer's instructions. Briefly, cells were seeded on coverslips, fixed, permeabilized and hybridized o.n. with anti-TRAP1 and anti-SDOS antibodies. Next day cells were hybridized with secondary antibodies conjugated with the PLA probes (PLUS and MINUS), and then subjected to ligation and rolling circle amplification using fluorescently labelled oligonucleotides. Cells were washed and mounted on slides using a mounting media with DAPI to detect nuclei and signal was detected by confocal microscopy analysis.

3.8. Cell fractionation

Mitochondria and ER were purified by using the Qproteome Mitochondria Isolation kit (Qiagen - 37612) according to the manufacturer's protocol. For the collection of ribosomal and non-ribosomal fractions, the lysates were centrifuged at $10,000 \times g$ at 4°C for 15 min in order to remove the mitochondria and cell debris. The supernatant was layered over a sucrose (20% wt/vol) cushion

containing cycloheximide and centrifuged at $149,000 \times g$ for 2 h. The pellet containing ribosomes and the upper and lower pellets of the non-ribosomal supernatants were collected. The ribosomal pellets were resuspended in the lysis buffer, after which immunoblotting was performed. Nuclear fractions were purified according to the manufacturer's protocol (Abcam).

3.9. *PNK assay*

Cells expressing eGFP-fusion proteins were UV-crosslinked on ice (150 mJ/cm^2), lysed (100mM KCl; 5mM MgCl₂, 10mM Tris pH 7.5, 0.5% NP40; 1mM DTT; protease inhibitor cocktail), and homogenized passing the lysate through a narrow needle (22G) followed by pulsed ultrasonication ($3 \times 10 \text{ s}$, 50% amplitude, on ice). Cleared lysates were treated with 50 U/ml DNaseI (Takara) and RNaseI for 15 min at 37 °C, and used for immunoprecipitation with GFP-Trap®_A agarose beads (Chromotek) for 2h at 4°C. Beads were washed four times with High salt buffer (500mM NaCl, 20mM Tris pH 7.5, 1mM MgCl₂, 0.05% NP40, 0.1% SDS, complete) and two times with PNK buffer (50 mM Tris pH 7.4, 50 mM NaCl, 10 mM MgCl₂, 0.5% NP-40, 5 mM DTT). RNA crosslinked to the tagged RBP is identified by radiolabeling with $0.1 \mu\text{Ci}/\mu\text{l}$ γ -32P ATP by T4 polynucleotide kinase (1U/ μl) in PNK buffer (50 mM NaCl, 50 mM Tris pH 7.5, 0.5% NP-40, 10 mM Mg₂Cl and 5 mM DTT) for 15 min at 850 rpm and 37°C. Beads were washed four to six times with PNK buffer and protein-RNA complexes were eluted by boiling samples 5 minutes at 95°C. Samples were analysed by SDS PAGE and autoradiography. For Flag-HA fusion proteins expressing cells the protocol described in⁹¹ was followed.

3.10. *Interactome capture for eGFP-tagged proteins*

1x15 cm plate of eGFP-fusion protein expressing cells was induced for 24 hrs (TRAP1) and 16 hrs (SDOS and eGFP) with $1 \mu\text{g/mL}$ doxycycline. TRAP1-eGFP and SDOS-eGFP cells were treated with $100 \mu\text{M}$ 4-thiouridine overnight and photoactivatable ribonucleoside-enhanced cross-linked (PAR-CL) on ice at 0.60 and 0.30 J cm^{-2} with UV light at 365 nm. Following UV-irradiation the protocol was performed as previously described (Strein C. et al., 2014).

3.11. Polysome profiling

3x10 cm plates of cells were incubated 15 min at 37°C with fresh medium supplemented with 100 µg/ml of cycloheximide (Sigma). Cells were then washed with ice cold PBS supplemented with 100 µg/ml cycloheximide and resuspended in 1 mL lysis buffer (10 mM Tris-HCl pH7.4, 100 mM KCl, 10 mM MgCl₂, 1% Triton-X100, 2 U/ml Turbo DNase (Ambion), 2 mM DTT, 10 U/ml Ribolock (Invitrogen), 100 µg/ml of cycloheximide). Glass beads (Sigma-Aldrich; G8772) were added to the lysate and cells were broken by vortexing at medium speed for 3 pulses of 10 s. After 5 min of incubation on ice, cell lysate was centrifuged for 5 min at 5000 rpm at 4°C. The supernatant was collected, and the absorbance was measured at 260 nm with the NanoDrop. Eight A260 units were loaded onto a 10-50% sucrose gradient obtained by adding 6 ml of 10% sucrose over a layer of 6ml 50% sucrose prepared in lysis buffer without Triton and containing 0.5 mM DTT, in a 12-mL tube (Polyallomer; Beckman Coulter). Gradients were obtained with the help of a gradient maker (Gradient Master, Biocomp). Polysomes were separated by centrifugation at 35000 rpm for 3 hrs using a Beckmann SW41 rotor. Eleven fractions of 1 mL were collected while polysomes were monitored by following the absorbance at 254 nm. Total protein was retrieved by 100% ethanol precipitation performed overnight and analysed by SDS-PAGE followed by Western blot.

3.12. Ribosome profiling

Ribosome profiling was performed according to the protocol described in (Ingolia N et al., 2012). Briefly, unfused eGFP, TRAP1-eGFP and SDOS-eGFP cells were cultured in 15 cm plates and induced with doxycycline for 24 hrs. After 15 min incubation with 100 µg/ml cycloheximide (Sigma-Aldrich; C4859) at 37°C, cells were washed with ice cold PBS and 1 mL of lysis buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM MgCl₂, 1 mM DTT, 100 µg/ml cycloheximide, 1% Triton-X100) was added. Cells were then collected and incubated on ice; glass beads (Sigma-Aldrich; G8772) were added to the lysate and cells were broken by vortexing at medium speed for 3 pulses of 10 s. After 10 min of incubation on ice, lysates were centrifuged for 10 min at 10000 rpm at 4°C, and the supernatant was recovered. RNA was partially digested with 3.5 µl of RNase I (100 U/µl, Invitrogen AM2294) per 800 µl of lysate. After 15

min of incubation at 24°C, lysates were placed on ice and supplemented with 10 µl of SUPERaseIn (20 U/µl, Invitrogen AM2694). Lysates were then loaded on a 34% sucrose cushion (34% sucrose in 20 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM MgCl₂, 1 mM DTT and 100 µg/ml cycloheximide) and monosomes were pelleted by centrifugation for 1 hr at 70000 rpm using a Beckman TLA 100.3 rotor. RNA was extracted from the pellet and ribosome protected fragments (RPFs) of 30 nucleotides were purified as described (Ingolia N et al., 2012). RPFs were depleted of ribosomal RNA with the Ribo-Zero rRNA removal kit (Epicentre MRZH116) according to manufacturer's indications. cDNA libraries were generated according to (Ingolia N et al., 2012) and sequenced by Solexa using a HiSeq 2000, Single Read, 50 nt at the CRG Genomics Core Facility, Barcelona, Spain.

3.13. *eCLIP-inspired individual nucleotide cross-linking and immunoprecipitation (iCLIP)-seq*

1x15 cm plate of eGFP-fusion protein expressing cells was induced for 24 hrs (TRAP1) and 16 hrs (SDOS and eGFP) with 1 µg/mL doxycycline. TRAP1-eGFP and SDOS-eGFP cells were treated with 100 µM 4-thiouridine overnight and photoactivable ribonucleoside-cross-linked on ice at 0.60 and 0.30 J cm⁻² with UV light at 365 nm. Immediately after irradiation, cells were lysed in 1 mL of lysis buffer (NaCl 100mM, MgCl₂ 5mM, Tris pH 7.5 10mM, NP40 0.5%, SDS 0.1%, Na deoxycholate 0.5%, DTT 1mM (fresh), 1x AEBSF (fresh). The cell lysate was passed 3 times through 27 1/2G needle and sonicated using a bioruptor (Digenode) for 3 cycles of 10 seconds (pause 15 seconds), level M at 4°C, then it was cleared by centrifugation at 17900g for 10 min at 4°C. RNA was then partially digested by adding 10 µl of 1:100 dilution of RNase I (Ambion, AM2295), as well as 2 µl of Turbo DNase (Ambion, AM 2238). After 3 min of incubation at 37°C under shaking at 1100 rpm 11 µl of Ribolock (Invitrogen) were added to each lysate. The lysates were precleared by incubation with 50 µL of equilibrated control agarose beads (Thermo Scientific) for 30 min at 4°C under gentle rotation. eGFP-fusion proteins were then captured from precleared lysates by incubation with 40 µL of GFP-Trap agarose beads (GFP-Trap_A, Chromotek) per mL of lysate for 2 h, 4°C, gentle rotation. Beads were collected by centrifugation and washed twice with High salt buffer

(NaCl 500mM, Tris HCL pH7.5 20mM, MgCl₂ 1mM, NP40 0.05%, SDS 0.10%, 1x AEBSF (fresh)); twice with Medium salt buffer (NaCl 250mM, Tris HCl pH7.5 20mM, MgCl₂ 1mM, NP40 0.05%, 1x AEBSF (fresh)) and twice with Low salt buffer (NaCl 150mM, Tris HCl pH7.5 20mM, MgCl₂ 1mM, NP40 0.01%, 1x AEBSF (fresh)). The RNA was dephosphorylated, and 3'-linker ligated as described in⁹². The protein/RNA complexes were isolated as described in⁹³. Samples were processed for subsequent steps as described in⁹². cDNA libraries obtained after PCR amplification with universal Solexa primers (25 cycles) were multiplexed and sequenced using an Illumina Next-generation sequencing platform at Science for Life Laboratory at Karolinska Institutet, Solna, Sweden.

3.14. RNA-seq from total RNA

Raw signal intensity data from Illumina HumanHT-12_V4_0_R2 microarrays normalized, batch effect removed, and low-quality annotation probes excluded. Differentially expressed genes (DEGs) and ranked (rnks) lists obtained by a moderated t-test on the linear model fit of the microarray data. DEGs with p-values <0.05 were retained. All the steps performed according to the “microarray analysis” best practice using R well known packages (R Core Team 2017; Ritchie ME. et al, 2015). Intersection of the DEGs performed according to the experimental design (same or different Fold Change sign).

3.15. GFP_trap immunoprecipitation and qPCR

1x15 cm plate of SDOS-eGFP and eGFP control cells was induced for 16 hrs with 1 µg/mL doxycycline. SDOS-eGFP cells were treated with 100 µM 4-thiouridine overnight and photoactivable ribonucleoside-cross-linked on ice at 0.30 J cm⁻² with UV light at 365 nm. eGFP cells were cross-linked on ice at 0.15 J cm⁻² with UV light at 254 nm. Immediately after irradiation, cells were lysed in 1 mL of lysis buffer (NaCl 100mM, MgCl₂ 5mM, Tris pH 7.5 10mM, NP40 0.5%, SDS 0.1%, Na deoxycholate 0.5%, DTT 1mM (fresh), 1x AEBSF (fresh), 100 U/mL Ribolock RNase inhibitor, 200 µM ribonucleoside vanadyl complex). The cell lysate was passed 3 times through 27 1/2G needle and sonicated using a bioruptor (Digenode) for 3 cycles of 10 seconds (pause 15 seconds), level M at 4°C, then it was cleared by centrifugation at 17900g for 10 min at 4°C. 50 µl of input were used

to measure fluorescence signal at plate reader in order to normalize the amount of eGFP proteins to be immunoprecipitated. 30 μ l of control magnetic agarose beads (Pierce) and GFP_trapMA beads (Chromotek) were equilibrated in Dilution buffer (NaCl 500mM, MgCl₂ 1mM, SDS 0.05%, NP40 0.05%, Tris pH 7.5 50mM, 100 U/mL Ribolock RNase inhibitor (fresh), 1x AEBSF (fresh)). Lysates were pre-cleared for 30 min under rotation at 4°C with 30 μ l of control magnetic agarose beads. GFP_trapMA were incubated with E. coli tRNA (1 mg/mL) for 15 min in dilution buffer under rotation at 4°C and then washed 2 times with dilution buffer. Pre-cleared lysates were then incubated with GFP_trapMA beads for 2 hrs under rotation at 4°C. Beads were then washed 2 times with High salt buffer (NaCl 500mM, Tris pH 7.5 20mM, MgCl₂ 1mM, NP40 0.05%, SDS 0.1%, Ribolock RNase inhibitor 100U/mL (fresh), 1x AEBSF (fresh) and 3 times with Low salt buffer (NaCl 150mM, Tris pH 7.5 20mM, MgCl₂ 1mM, NP40 0.01%, Ribolock RNase inhibitor 50U/mL). Beads were resuspended in 100 μ l of Proteinase K buffer (NaCl 0.1M, Tris pH 7.5 10mM, EDTA 1mM, SDS 0.5%, 200 μ g/mL Proteinase K, 50 pg spike-in control RNA) and incubated at 55°C for 1 hr under constant mixing. To recover RNA, 100 μ l of TRI Reagent were directly added to the buffer-containing beads followed by extraction and ethanol precipitation. The RNA was reverse transcribed, and the resulting cDNA was analysed by quantitative PCR. The amount of precipitated RNA from IPs was normalized to the amount of the spike-in control.

3.16. Protein-protein interaction identification by MS

1x15 cm plate of TRAP1-eGFP, SDOS-eGFP and unfused eGFP expressing cells was induced for 24 hrs with 1 μ g/mL of doxycycline. Cells were then lysed with 1 mL of lysis buffer (NaCl 150 mM, Tris-HCl pH7.5 10 mM, Triton X-100 1%, MgCl₂ 5 mM, DTT 5mM (fresh) and AEBSF 1x (fresh)) on ice for 15 min. Lysates were cleared by centrifugation at 16000g for 5 min. Lysates were precleared by incubation with 50 μ L of equilibrated control agarose beads (Thermo Scientific) for 30 min at 4°C under gentle rotation. eGFP-fusion proteins were then captured from precleared lysates by incubation with 40 μ L of GFP-Trap agarose beads (GFP-Trap_A, Chromotek) per mL of lysate for 2 h, 4°C, gentle rotation. Beads were collected by centrifugation and washed six times with lysis buffer. Samples were eluted from the beads by pH elution as indicated in the manufacturer's

protocol (GFP_trapA; Chromotek) and sent to the MS facility of Department of Biochemistry, University of Oxford, for the analysis.

Bioinformatic Methods

3.17. *Bioinformatic analysis of iCLIP datasets*

Sequencing of the iCLIP libraries (75bp single-end reads) was carried out on an Illumina NEXTseq at the Karolinska Institutet, Solna, Sweden. We used the demultiplex script from the iCount pipeline (Curk et al., 2016, <http://icount.readthedocs.io/en/latest/>) to trim off adapter sequences, to extract sample and molecular barcodes, and to demultiplex. We then mapped the reads to the human genome and annotation version GRCh38 downloaded from ENSEMBL using splice-aware mode of the STAR aligner (Dobin et al., 2012) through the iCount script mapstar. We then removed the PCR duplicates and identified the crosslinked sites on RNA using xlsites and peaks from the iCount pipeline. The crosslinked base is identified as the last base of the cDNA and first base of the read after the barcodes in this protocol. We then defined binding regions on RNA using a window of 10nt with BEDTools (Quinlan and Hall, 2010). Binding regions were then assigned to genes and transcript features based on their overlap with annotated transcript features (ENSEMBL version GRCh38) utilising R/Bioconductor package GenomicRanges (Lawrence et al., 2013). We used the following assignment preference rule to assign those peaks that overlapped multiple annotated features at a gene level: protein coding exonic > miRNA > snRNA > snoRNA > rRNA > lincRNA > antisenseGenes > protein coding intronic > other > senseIntronic. Peaks overlapping multiple annotated exonic protein coding features were further assigned to transcript features according to the following assignment preference rule: CDS > 5UTR > 3UTR > other. In order to define reliable target genes for downstream analyses and experimental validation, we selected binding region with a False Discovery Rate < 0.05 and for which there was no signal detected for the gene in the negative control (eGFP) sample.

3.18. *Bioinformatic analysis of Ribosome Profiling datasets*

Raw sequence reads were demultiplexed and adapter remnants were trimmed using cutadapt (Martin M., 2011).

Reads derived from rRNAs were filtered out after a first pass mapping using bowtie2 and a custom composite rRNA genome. Remaining

reads were aligned with Tophat2 on the hg38/GRCh38 human genome and the corresponding ENSEMBL transcriptome. Number of reads mapping “exon” was calculated with htseq-count (Anders S et al., 2015) according to ENSEMBL annotation. Differentially expressed Ribosome Protected Fragments (RPFs) were calculated using the DEseq2 bioconductor R package (Love MI et al., 2014). For downstream analysis, we considered only RPFs with Fold Change > 3.0. Selected RPFs were normalized to the Microarray results in order to select only those genes regulated at translational level.

3.19. Gene Ontology Analyses

iCLIP and RP data (significantly impacted pathways, biological processes, cellular component, diseases) were analysed using Advaita Bio’s iPathwayGuide (<http://www.advaitabio.com/ipathwayguide>). To highlight a possible activation/deactivation of biological functions and pathways the gene set enrichment analysis (GSEA) (Subramanian A. et al, 2005) of DEGs was performed for *rnk* each list. In particular, the analysis focused on hallmark, c2 and c5 gene sets collected by the Molecular Signatures Database (MSigDB) (Liberzon A. et al, 2015)

List of Oligos

Oligo name	Oligo sequence (5'-3')
CC2D2A FW	AGGGGCATCTCCAACACTCA
CC2D2A RV	TCATTTTGGCTGGGATGGGT
TMEM67 FW	TGCCCAGAAAACATGAAAGGTG
TMEM67 RV	TCACAGAGCTCACAAGTTGCT
NPHP1 FW	TTGGAGCAGCCTGATGTGAT
NPHP1 RV	CTCTTCTTCTGCCCACCTGAA
KIF7 FW	ATGGAAACCTGACTGCCTGG
KIF7 RV	CAGTGGTTCGAACCTCGCAGG
TTC21B FW	GGCGAGACAATCCCCAGAG
TTC21B RV	CCTGAAGACTGGATCACTTCCA
ZNF423 FW	CATGGATGCATAAGAAGAGGGTTGA
ZNF423 RV	TGGTTTTCTGATCGCACTCTGG
RPGRIP1L FW	CATTTCCCAGGAGGCTACGG
RPGRIP1L RV	GTTGGACCAGACATGGCCTA

AHI1 FW	GTGTCAACCTGGGGCTGAAT
AHI1 RV	ATGCAGAGGACTGAGAATGCAA
TCTN1 FW	GCACAACTGAGCAAGACTGC
TCTN1 RV	AAAGGGGCCACGTAATCTGG
TOPORS FW	CGACACCGACCTAGCTTTCT
TOPORS RV	GCTAGTGCCAGCTTTAGGTGA
TMEM107 FW	CCCCGAGGAGTATGACAAGC
TMEM107 RV	GCACTACAGTGAGCCCCAAT
LUC FW	TACAACACCCCAACATCTTCGA
LUC RV	GGAAGTTCACCGGCGTCAT
ACTB FW	CCTCACCCCTGAAGTACCCCA
ACTB RV	TCGTCCCAGTTGGTGACGAT

4. RESULTS

4.1. SDOS is a novel TRAP1-interacting partner

A previous mass spectrometry analysis performed to identify the protein partners of TRAP1 in a proteome-wide manner allowed to identify, among the others, several mitochondrial and cytoplasmic proteins involved in different pathways, such as protein synthesis and post-translational modifications, cell cycle regulation, cell metabolism, trafficking and mRNA synthesis, transport and modification⁸⁷. Among those, coherently with the aim of the study of further dissecting the molecular mechanisms involved in TRAP1 regulation of its substrates, we focused our attention on the putative RNA-binding protein SDOS. In order to confirm that SDOS is indeed a TRAP1-interacting partner, we performed co-immunoprecipitation analysis. To this aim, TRAP1 was immunoprecipitated from HCT116 cells 24 hrs after transfection of a SDOS-myc expressing construct. Results showed a specific co-IP band following blot with anti-myc antibody (Fig. 1A). TRAP1/SDOS interaction in HCT116 cells was further confirmed by proximity ligation assay, as shown in Fig. 1B.

For our subsequent analyses, we took advantage of the “Flp-In™ T-REx™” (FITR) System, which allows the generation of stable mammalian cell lines exhibiting tetracycline-inducible expression or silencing of a gene of interest from a specific genomic location, thus ensuring the integration in each cell of the population.

Once established HeLa FITR cell lines expressing the eGFP-fusion proteins of interest, we performed a mass spectrometry analysis to confirm SDOS as a TRAP1-interacting partner in our model, and to ensure that the fused eGFP protein does not interfere with this interaction. TRAP1-eGFP and SDOS-eGFP were immunoprecipitated - immunoprecipitation of the unfused eGFP was used as a negative control - and analysed by mass spectrometry to look at the protein-protein interaction. SP3 sample preparation method was used to maximise sensitivity, while “label free” quantification was used to compare the unfused eGFP control to the protein of interest. Results of the analysis demonstrated not only that SDOS is one the strongest TRAP1-interacting partners, but that TRAP1 was one of the strongest SDOS-interacting partner as well (Table 1). Neither TRAP1 nor SDOS were found in the eGFP negative control sample. Moreover, the analysis showed 53BP1 protein as the major interactor of SDOS,

Results

according to recent works by other groups^{86,88} and the ribosomal protein S28 as a protein-partner common to TRAP1 and SDOS, suggesting that both associate with ribosomes.

SDOS-eGFP partners		
Gene name	t-test SDOS-eGFP/eGFP (-log10)	Gene ID
NUDT16L1	12,0802	Q9BRJ7
TP53BP1	11,0722	Q12888
LGALS3BP	9,86244	Q08380
TUBB8	9,00189	Q3ZCM7
NUDT16	8,05465	Q9BRJ7
SQSTM1	7,90479	Q13501
TUBA1A	7,84447	Q71U36
CCT4	7,6541	P50991
DYNLL1	7,46163	P63167
USP11	7,29486	P51784
TRAP1	6,92604	Q12931
CCT8	6,83071	P50990
SLC7A5	6,46204	Q01650
HNRNPH3	6,1067	P31942
BAG2	6,02968	O95816
TCEAL1	6,00085	Q15170
CCT5	5,88357	P48643
MIF	5,75599	P14174
SLC25A10	5,34728	Q9UBX3
TUBA4A	4,91643	P68366
RPS28	4,91507	P62857
SLC16A3	4,88154	O15427
PPP6R3	4,82287	Q5H9R7
LAMA1	4,40167	P25391
POLDIP2	4,22019	Q9Y2S7
ANKRD28	4,00495	O15084
PPP6C	3,92096	O00743

TRAP1-eGFP partners		
Gene name	t-test TRAP1-eGFP/eGFP (-log10)	Gene ID
ABCF2	14,2995	Q9UG63
TRAP1	12,6434	Q12931
TOMM40	11,2559	O96008
ACAT1	8,15674	P24752
HSP90AB1	6,42103	P08238
MIF	5,29286	P14174
NUDT16L1	4,57307	Q9BRJ7
POLDIP2	3,89041	Q9Y2S7
RPS28	3,7948	P62857

Table 1 Complete list of SDOS and TRAP1 protein-partners identified by mass spectrometry analysis. Gene name, t-test value (-log10) and Gene ID were reported for each protein-partner. Relevant protein-partners were highlighted in color. TRAP1 and NUDT16L1 (red) were found in each-other list. RPS28 (blue) was found in both lists.

To confirm TRAP1/SDOS interaction in HeLa cells, we performed immunoprecipitation (IP) of a myc-tagged SDOS upon transfection in both HeLa FITR sh-eGFP and sh-TRAP1 cells. TRAP1 silencing was induced for 48 hrs followed by 24 hrs of SDOS-myc transfection. HeLa sh-TRAP1 cells were used as negative control and the IPs were analysed by western blot. As shown in Fig. 1C SDOS-myc was

immunoprecipitated with Myc-Trap® agarose beads which utilizes small recombinant antibody fragments covalently coupled to the surface of agarose beads recognizing the Myc-tag sequence EQKLISEEDL at the N-terminus, C-terminus, or internal site of the fusion protein. Immunoblot with anti-TRAP1 antibody revealed a TRAP1-immunoreactive band in the SDOS-myc IP from the sh-eGFP cells, whereas no bands was detected in the sh-TRAP1 negative control, where the lack of TRAP1 caused a loss in the co-immunoprecipitated TRAP1 protein.

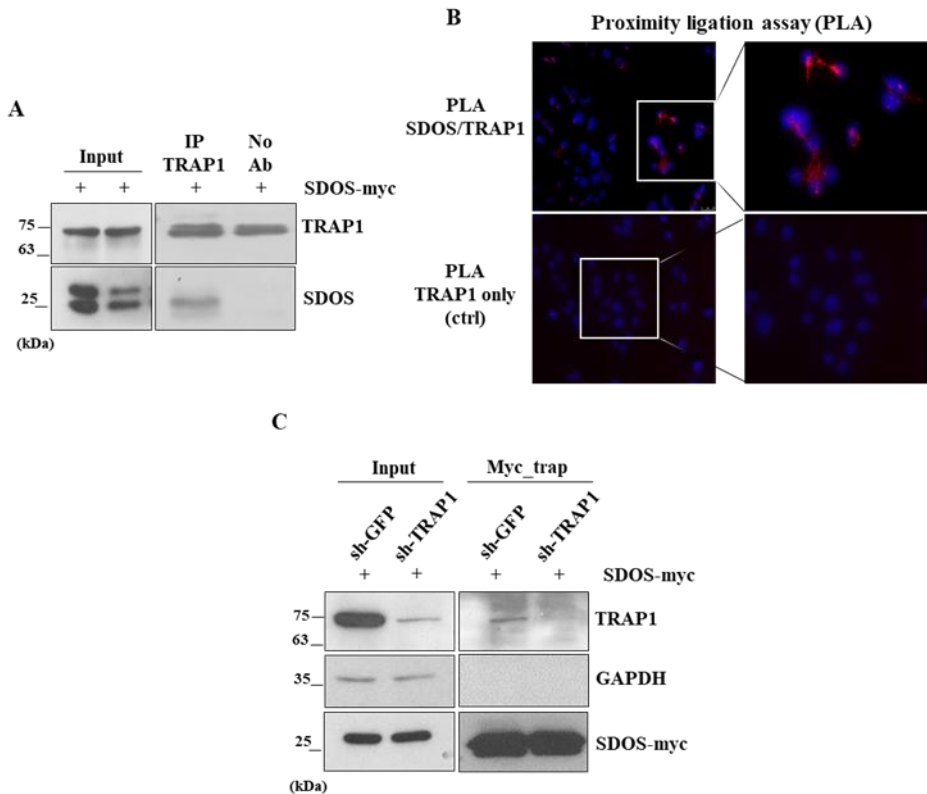


Figure 1 TRAP1 and SDOS are interacting partners. (A) Total HCT116 lysate was immunoprecipitated using α -TRAP1 antibody following 24 hrs of SDOS-myc transfection and immunoblotted using the indicated antibodies. No Ab, total cellular extracts incubated with A/G plus agarose beads with normal mouse IgG. (B)

Representative image of proximity ligation assay showing the interaction of SDOS with TRAP1 in HCT116 cells. Positive signals of interaction are shown as red dots, nuclei are stained with DAPI (blue). (C) HeLa sh-eGFP and sh-TRAP1 cells were induced for 48 hrs. Total lysates were immunoprecipitated with Myc_trap agarose beads following 24 hrs of SDOS-myc transfection and immunoblotted with indicated antibodies.

4.2. SDOS localizes in the cytosol and the ER, where it interacts with TRAP1

At the beginning of my PhD, SDOS was essentially a poorly characterized protein identified in the cytosol where it interacts with Syndecan4 and Paxillin⁸⁵. Only recently it has been demonstrated that it is involved in a specific mechanism of DNA repair with 53BP1 in the cell nucleus⁸⁶. Therefore, I decided to further investigate its localization within our cellular model through a sub-cellular fractionation of both HeLa WT and HeLa SDOS-eGFP cells. WB analysis of sub-cellular compartments confirmed the cytosolic localization of both SDOS-eGFP and the endogenous SDOS, but more importantly demonstrated that SDOS localizes on the ER. TRAP1 mitochondrial and reticular localization were also confirmed (Fig. 2A-B). Moreover, WB analysis performed on the nuclear fraction from HeLa SDOS-Flag-HA cells extracts, showed that SDOS localizes in the nucleus as well (Fig. 2C).

The evidence that both TRAP1 and SDOS localize in the ER prompted us to hypothesize that these proteins might interact in this specific cellular compartment. Therefore, we performed a TRAP1 IP from the ER fraction of both sh-eGFP and sh-TRAP1 HeLa cells, following 96 hrs of induction. Western blot analysis with SDOS antibody demonstrated that SDOS localizes on the ER as well - as indicated by the bands identified in the ER fraction - and that TRAP1/SDOS interaction occurs in this compartment, since a specific band immunoreactive to anti-SDOS antibodies was detected in the IP from the sh-eGFP cells but not in the sh-TRAP1 cells, used as a negative control (Fig. 2D).

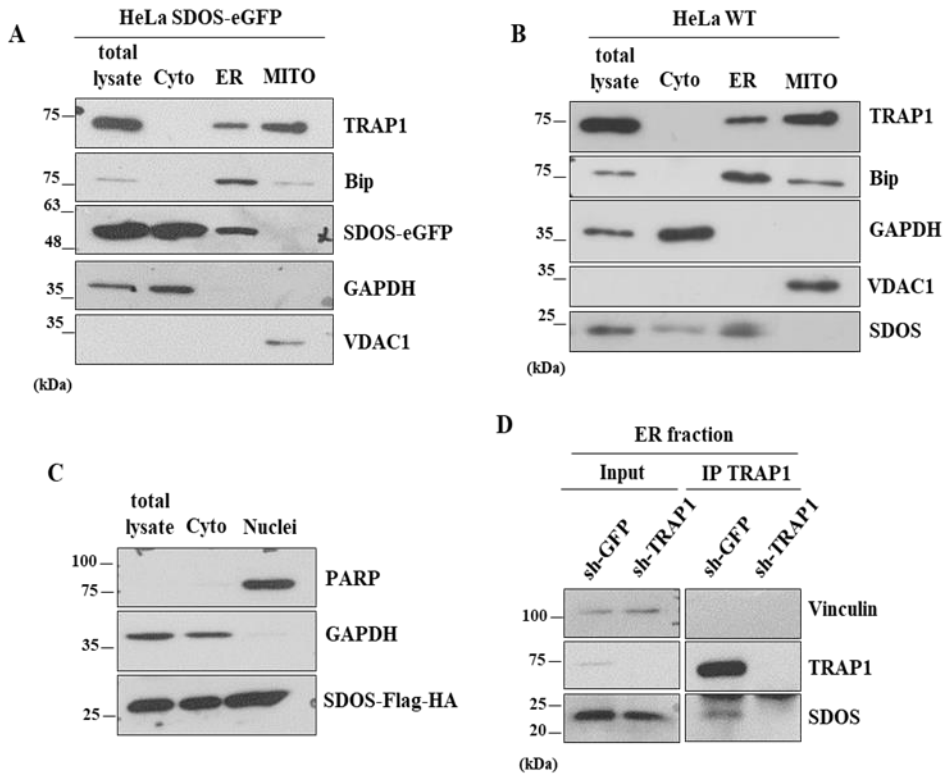


Figure 2 SDOS interacts with TRAP1 on the ER and localizes in both the cytoplasm and the nucleus. (A) and (B) Total HeLa SDOS-eGFP and HeLa WT lysates were fractionated into mitochondrial (MITO), cytosolic (Cyto) and microsomal (ER) fractions as described in Materials and Methods, separated by SDS-PAGE and immunoblotted with α -SDOS and α -TRAP1 antibodies. The purity of the fractions was assessed by using α -GAPDH, α -BiP, α -VDAC1 antibodies, specific for each subcellular compartment. (C) Total HeLa SDOS-Flag-HA lysate was fractionated into cytosolic and nuclear fractions as described in Materials and Methods and immunoblotted with α -SDOS antibody. α -PARP and α -GAPDH antibodies were used to assess purity of the fractions. (D) TRAP1 and SDOS co-IP analysis on the microsomal fraction (ER), obtained as described in Materials and Methods. WB of immunoprecipitates was performed by using the indicated antibodies.

4.3. SDOS associates with actively translating polyribosomes in cancer cell

Previous studies from our group demonstrated that TRAP1 role in protein synthesis is linked to its association with the translational apparatus, including ribosomes⁷⁴. Starting from this observation and from the evidence that among the protein partners identified by mass spectrometry the ribosomal protein S28 was found as common to both TRAP1 and SDOS (Table 1), we hypothesize that SDOS might associates with ribosomes as previously demonstrated for TRAP1. Therefore, we isolated ribosomal fraction from HCT116 cell extracts through ultracentrifugation on a sucrose cushion. Interestingly, WB analysis of the fractions demonstrated that SDOS associates with ribosomes, as previously demonstrated for TRAP1 (Fig. 3A). The same result was confirmed upon expression of SDOS-myc in HCT116 cells (Fig. 3A).

To verify if SDOS association with ribosomes might indicate an influence on protein synthesis, we performed a polysome profiling analysis, in collaboration with Dr. Elias Bechara at CRG in Barcelona, by ultracentrifugation on sucrose gradients of total extracts of cells expressing our eGFP-fusion proteins. SDOS-eGFP and unfused eGFP proteins expression were induced for 24 hrs and cells were treated with cycloheximide (CHX) before lysis to stabilize ribosomes on mRNAs. The analysis of polysome profiles upon SDOS overexpression showed a slight reduction in the amount of active polyribosomes compared to the eGFP-expressing control cells, as shown in Figure 3B.

Moreover, monosome and polysome fractions were collected from the gradient and analysed by western blot. Results demonstrated for the first time that SDOS associates with active polyribosomes, supporting the hypothesis of a role for this protein in mRNA translation (Fig. 3C).

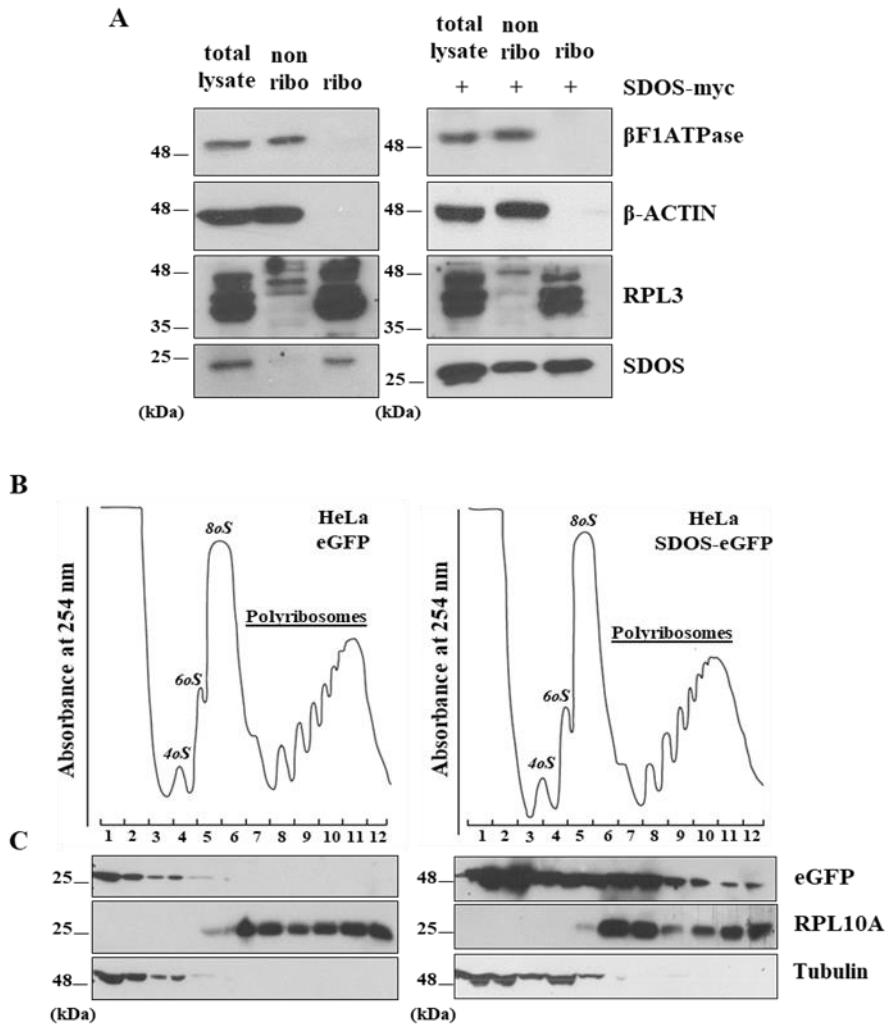


Figure 3 SDOS associates with and decreases active polyribosomes. (A) Ribosomal purification from HCT116 cells and HCT116 cells transfected with SDOS-myc, followed by immunoblot with α -SDOS on Ribosomal (ribo), non-ribosomal (non-ribo) and total lysate fractions. The purity of the fractions was assessed by using α -RPL3, α - β Actin and α - β F1ATPase antibodies. (B) Separation of cytoplasmic extracts from eGFP and SDOS-eGFP cells was performed by ultracentrifugation on sucrose gradients as described in Materials and Methods. The absorbance profile, measured at 254 nm, indicates the sedimentation of the particles: fractions 1 and 2 free cytosolic proteins or light complexes; fractions from 3 to 5 ribosomal subunits (60S, 40S) and monomer (80S); fractions from 6 to 12

polysomes. (C) Proteins from the fractions were analysed by western blot with the indicated antibodies.

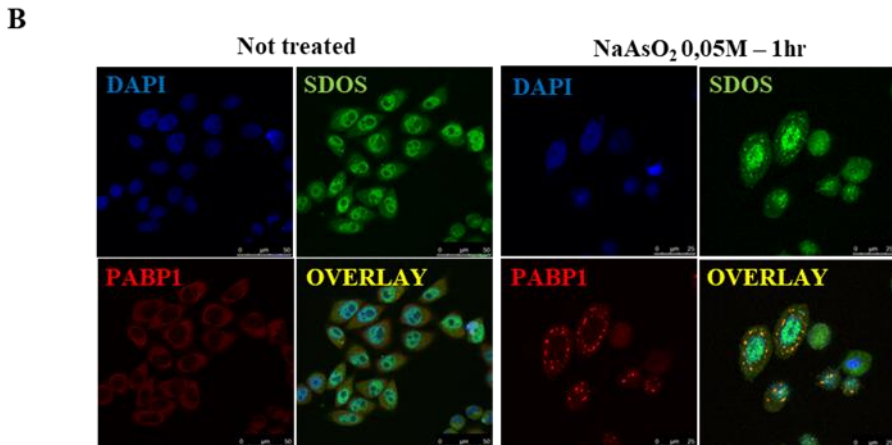
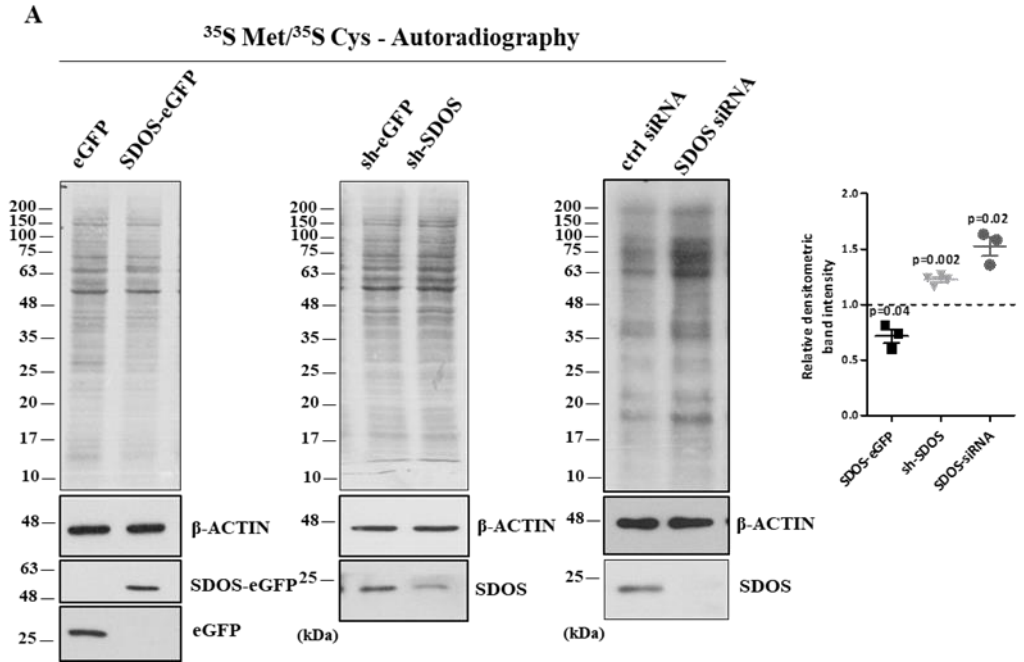
4.4. SDOS influence global protein synthesis and takes part to stress granules

To confirm that SDOS might influence mRNA translation, we decided to monitor protein synthesis by radioactive labelling of newly synthesized proteins followed by autoradiography, either upon overexpression or silencing of SDOS in HeLa cells, and upon silencing by SDOS-directed siRNA in HCT116 cells. As shown in Fig. 5A, results confirmed the analysis of polysome profiling showing that SDOS-eGFP cells incorporate less radioactive amino acids than the eGFP control. Accordingly, SDOS silenced cells incorporate more radioactively-labelled amino acids than the relative control and this phenotype is observed in HCT116 cells as well, following SDOS transient silencing by a specific siRNA. Densitometry-based quantification and statistical significance of this analysis is reported in the right panel of Fig. 5A. Taken together, these data demonstrated that SDOS is able *per se* to influence global protein synthesis.

Starting from the evidence that among the SDOS protein partners identified by MS there are components of stress granules (SGs) like CCT4, CCT5 and CCT8⁸⁹, we wondered if SDOS might take part to these mRNP aggregates, being involved in the well-known cross-talk between mRNA translation and degradation⁹⁰. To test this hypothesis, we performed immunofluorescence (IF) analysis on HeLa SDOS-Flag-HA cells, upon sodium arsenite (NaAsO₂) treatment, a well-known inducer of SGs, using polyA binding protein 1 (PABP1) antibody as a SG marker. As shown in Fig. 5B, NaAsO₂ treatment induced formation of PABP1-containing aggregates which co-localized with SDOS. HeLa SDOS-Flag-HA untreated cells were used as negative control. To further characterize the role of SDOS in these subcellular compartment, we performed RNA fluorescence *in situ* hybridization (FISH) analysis using fluorescently-labelled oligo (dT) as probe against mRNA polyA-tails following NaAsO₂ treatment. Results showed co-localization of polyadenylated RNA with SDOS-eGFP (Fig. 5C), thus supporting the RNA-binding capacity of SDOS. eGFP unfused control cells were used as negative control showing no co-localization. These IF analyses also confirmed SDOS nuclear localization as above demonstrated by cell fractionation and WB.

Results

Taken together, these results suggest that SDOS might regulate mRNAs sorting and processing, for either re-initiation of translation or degradation, by taking part of SGs.



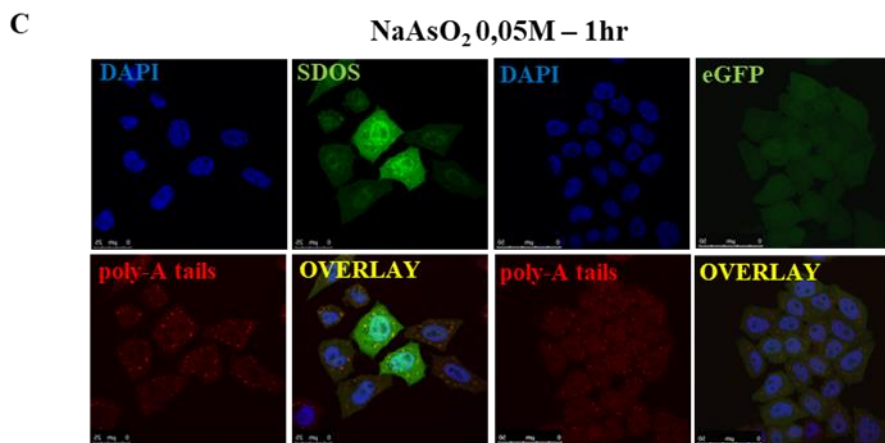


Figure 5 SDOS affects mRNA translation by taking part of SGs. HeLa eGFP and SDOS-eGFP were induced for 24 hrs; HeLa sh-GFP and sh-SDOS cells were induced for 48 hrs, while HCT116 cells were transfected with control or SDOS-directed siRNA for 48 hrs. **(A)** Cells were incubated in cysteine/methionine-free medium containing 50 μ Ci/ml ³⁵S-labeled cysteine/methionine for 30 min and washed with PBS. Lysates were collected, subjected to SDS-PAGE and analyzed by autoradiography. α - β Actin was used to normalize the results. Immunoblot with α -SDOS and α -eGFP antibodies was performed to verify SDOS silencing and SDOS-eGFP overexpression. Densitometric band intensities was calculated for 3 replicates by assuming protein levels of the control equal 1. Numbers above bars indicate the statistical significance (P-value), based on one-sample t-test. **(B)** Immunofluorescence analysis showing co-localization of PABP1 (red) with SDOS-Flag-HA (green) upon NaAsO₂-mediated SG induction. **(C)** HeLa eGFP and SDOS-eGFP were induced for 16 hrs and subjected to RNA-FISH analysis, which shows co-localization of RNA (red), stained by using fluorescently-labelled oligo (dT) as probes, with SDOS-eGFP (green).

4.5. SDOS and TRAP1 have RNA-binding capacity

TRAP1 role in translation was deeply investigated in our studies, however, as previously stated, mechanisms of substrates recognition are still unknown. Starting from the observation that molecular chaperones can have RNA-binding properties⁹¹ and that RBPs are involved in mRNA localization and translation⁴⁹, our hypothesis is that TRAP1 might recognize its substrates as mRNAs, through a direct binding or through its interaction with putative RBP-interacting partners such as SDOS.

Therefore, we combined a dual approach to demonstrate that TRAP1 and/or SDOS are RNA-binding proteins. First, we performed a

polynucleotide kinase (PNK) assay and then a eGFP-based RNA-binding assay. As for the PNK assay, HeLa F1TR expressing the eGFP-fusion proteins, were induced and directly irradiated with UV light, which forms covalent bonds between protein and RNA that are in direct contact. Protein-RNA complexes were immunoprecipitated with GFP-Trap® agarose beads and treated with different concentration of RNaseI. At low concentration of RNase, the protein-RNA complexes appear as a smear because of the high molecular weight generated by long stretches of RNAs causing a shift on the electrophoretic mobility of the protein. Increasing concentrations of RNase allow only short fragments to remain bound to the protein thus allowing, following T4 polynucleotide kinase catalysis and autoradiography, the identification of a single band corresponding to the target protein bound to the ³²P-labeled RNAs fragments. WB analysis with anti-eGFP antibody performed on the same membrane then allows to verify that the identified band belongs to the proteins of interest. Our results demonstrated, for the first time, that both TRAP1 and SDOS are novel RNA-binding proteins (Fig. 6A). In this experiment MOV10-YFP and unfused eGFP expressing cells were used as positive and negative control, respectively.

To further demonstrate that the presence of the eGFP-fusion protein does not influence the RNA-binding properties, this assay was replicated with the Flag-HA-tagged proteins, obtaining the same results (Fig. 6B).

Then, we approached this aspect in a reverse prospective, by performing a small-scale interactome capture. In brief, eGFP-fusion proteins expressing HeLa cells were directly irradiated with UV light and the RNA bound to the proteins was captured with oligo-dT beads. Following stringent washes, green fluorescence in eluates was measured to quantify RNA-binding. As shown in Fig. 6C, results confirmed the RNA-binding capacity of both TRAP1 and SDOS - according to what seen in the PNK assay. In this case, hnRNPC, a known RBP, has been used as positive control. Unfused eGFP expressing cells, whose eluates measurement gave no signal, were used as negative control.

The use of these complementary approaches strongly demonstrated that TRAP1 and SDOS are novel, non-canonical, RBPs.

Results

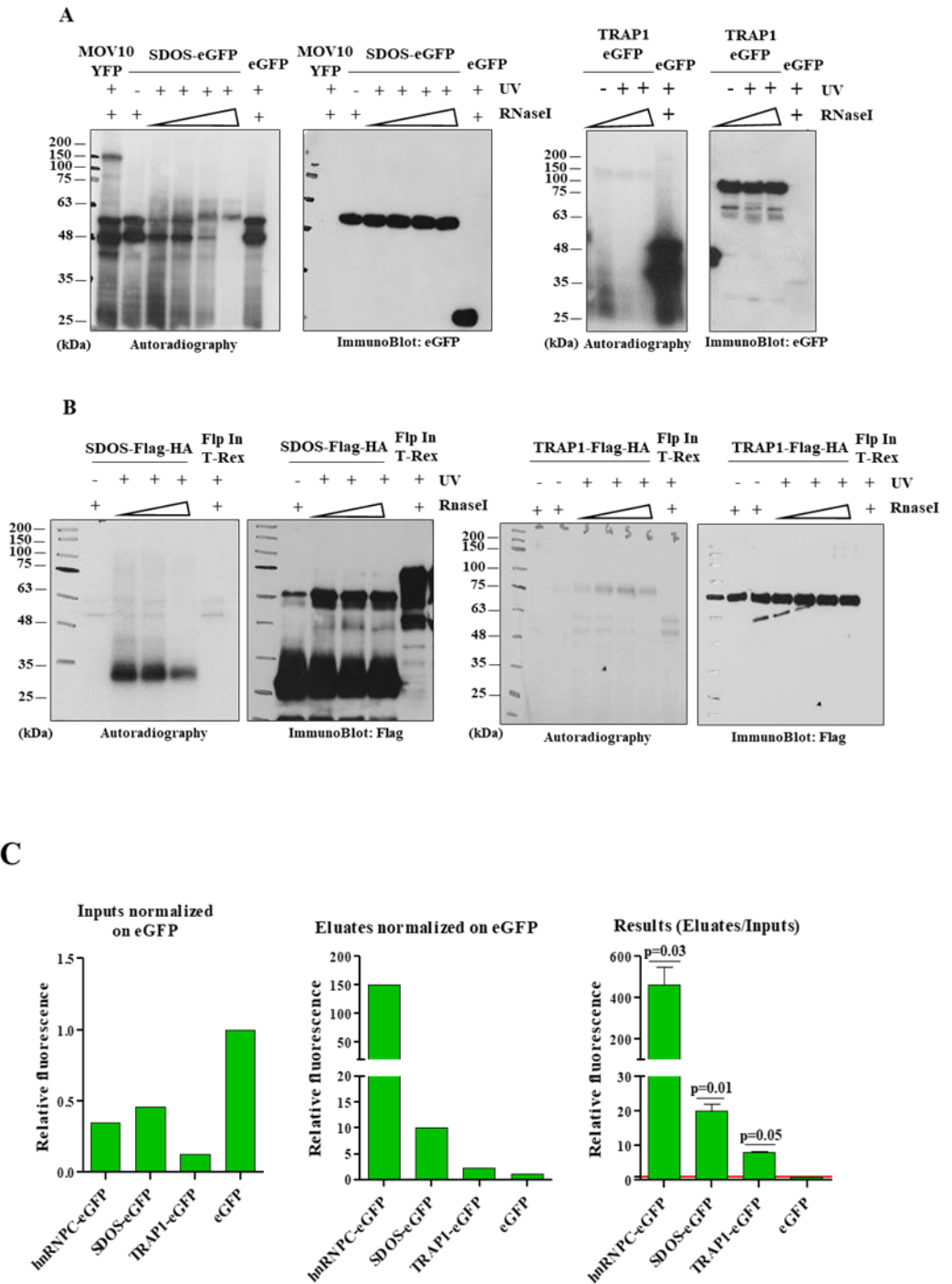


Figure 6 TRAP1 and SDOS are novel, non-canonical, RNA-binding proteins.

(A) and (B) HeLa FITR cells were induced for 16 hrs to induce the expression of the fusion proteins and directly irradiated with UV light, which forms covalent bonds between protein and RNA that are in direct contact. Protein-RNA complexes were immunoprecipitated either with GFP_trap agarose beads or M2-FLAG magnetic agarose beads and treated with different concentration of RNaseI. Following ³²P labelling of RNA with T4 polynucleotide kinase catalysis, the IPs were subjected to SDS-PAGE and autoradiography in order to visualize the target protein-labeled RNA complexes. Immunoblot with α -eGFP or α -FLAG antibodies were performed to confirm that the revealed band correspond to the protein of interest. (C) HeLa FITR cells were induced for different time points to induce the expression of the fusion proteins and with 100 μ M 4-thiouridine overnight to promote PAR-CL. Following *in vivo* UV crosslinking, oligo(dT) capture, and stringent washes, green fluorescence in eluates and inputs was measured to quantify TRAP1-eGFP and SDOS-eGFP RNA binding. Unfused eGFP was used as negative control and the well-established RNA-binding protein hnRNP-eGFP as a positive control for RNA binding.

4.6. Identification of SDOS and TRAP1 directly regulated targets at translational level by eCLIP-inspired iCLIP and Ribosome Profiling

Starting from the evidence that RBPs play fundamental roles in mRNA translation, we hypothesized that TRAP1 and SDOS RNA-binding properties might be important for their role in this process. Therefore, we decided to perform three high throughput analyses - individual nucleotide resolution cross-linking and immunoprecipitation-sequencing (iCLIP-seq), Ribosome Profiling-sequencing (RP-seq) and Differential Gene expression analysis (GE) - to identify those targets that are 1) directly bound at RNA level by TRAP1 and/or SDOS and 2) regulated at translational level.

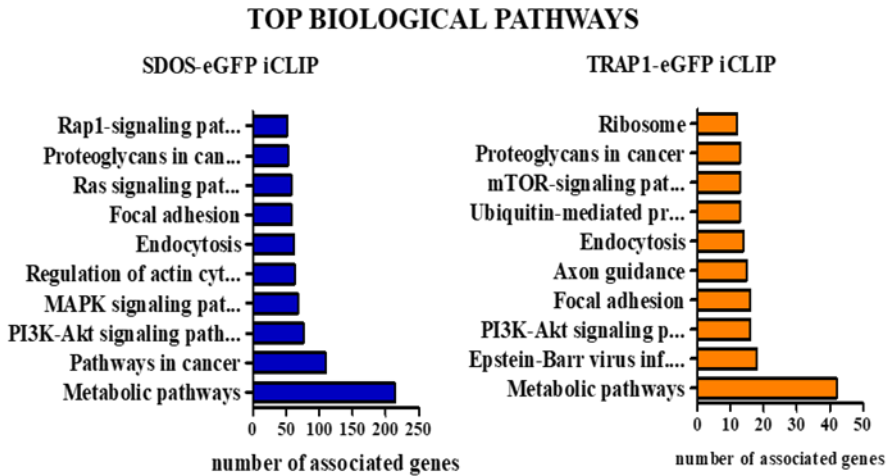
4.6.1. eCLIP-inspired iCLIP-seq

To identify RNAs that are SDOS and TRAP1 direct targets, we performed a slightly modified version of the iCLIP-seq protocol from the eGFP-fusion proteins expressing HeLa cells. After UV *in vivo* cross-linking, SDOS-eGFP, TRAP1-eGFP and unfused (control) eGFP proteins were immunoprecipitated with GFP_trap agarose beads and subjected to SDS-PAGE to isolate protein-RNA complexes. Standard iCLIP protocol relies on the radioactive labelling of the protein-bound RNA in order to visualize it by autoradiography and avoid non-specific products to be isolated⁹². We omitted this step as previously described in the eCLIP protocol⁹³ and isolated the RBP-

RNA complexes on the basis of the predicted RBP-IP molecular weight observed by WB. The RNA was then isolated, reverse transcribed and sequenced by Next-Generation sequencing. We performed three independent iCLIP analyses that show poor correlation due to the variability of the experiment. Therefore, we decided to analyse all the libraries and to consider positive all the targets showing a False Discovery Rate (FDR) <0.05 and specificity for the target protein (no peaks detected in the eGFP negative control). This cut-off reveals a set of 4453 targets for SDOS-eGFP and 687 targets for TRAP1-eGFP respectively.

Interestingly, a gene ontology (GO) analysis performed on these lists showed that some of the top biological pathways enriched in the iCLIP data are common to both TRAP1 and SDOS. Among them, metabolic pathways, PI3K-Akt signaling pathway, focal adhesion, proteoglycans in cancer, endocytosis (Fig. 7A). This is of particular interest for the hypothesis that SDOS and TRAP1 might share functional roles, based on the relevant evidence that TRAP1 and SDOS are among the top protein partners of one another. On one hand, we demonstrated that TRAP1 is responsible for a metabolic rewiring in ovarian cancer cells which, in turn, is responsible for the inflammation-induced platinum resistance⁶⁹. Moreover, our previous works demonstrated that TRAP1 affects cell migration through a regulation of the PI3K-Akt axis⁷⁶. On the other hand, it was demonstrated that SDOS is involved in the assembly of focal adhesions through its interaction with Paxillin and Syndecan-4⁸⁵. Therefore, it will be interesting to verify if SDOS and TRAP1 might work together in one (or both) of these shared pathways in the near future. Besides, analysis for disease-associated genes of SDOS iCLIP data showed enrichment of genes mostly responsible for neurological disorder, which can be caused by aberrant accumulation of SGs¹⁰⁰, thus supporting our evidence about a role for SDOS in these subcellular compartments, and ciliopathies, which are commonly caused by defect in the primary cilia⁹⁴ (Fig. 7B). Among them, Joubert Syndrome (JBS), whose associated-genes were also enriched in TRAP1 iCLIP data. Furthermore, analysis for disease-associated genes of TRAP1-iCLIP showed enrichment of genes involved in several types of cancer. Among the others, colorectal and ovarian cancer, which are consistent with the well-characterized role of TRAP1 in these cancer types^{65,69} (Fig. 7B).

A



B

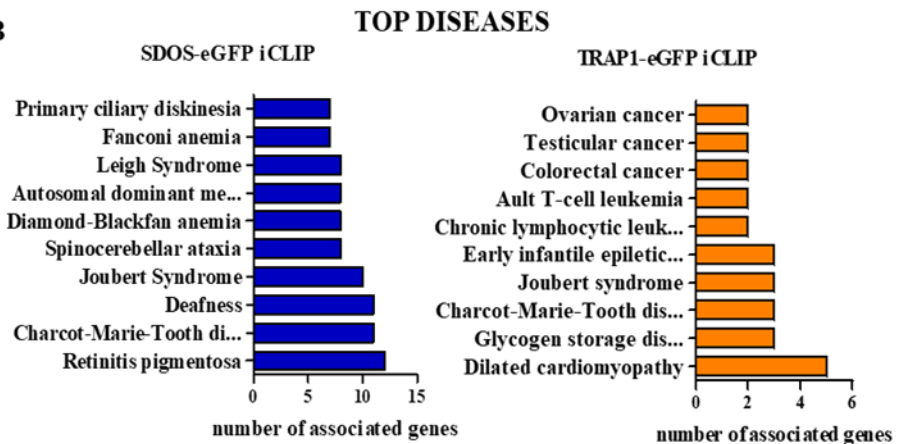


Figure 7 SDOS and TRAP1 iCLIP targets share common biological pathways. (A) and (B) Analyses of biological pathways and diseases-associated genes from SDOS and TRAP1 iCLIP data using Advaita Bio's iPathwayGuide.

4.6.2. Ribosome Profiling-seq and Gene expression

In order to identify those targets regulated by SDOS and/or TRAP1 at translational level, we performed Ribosome Profiling (RP)-sequencing from our eGFP-fusion proteins expressing HeLa cells. This technique allows to obtain quantitative information about translation by deep sequencing of ribosome-protected fragments (RPFs) upon CHX treatment, which stabilizes ribosomes on the mRNAs, that correspond to the actively translated mRNAs⁹⁵. In brief, RNA was extracted from isolated ribosome and RPFs were selected, by cutting the corresponding gel bands between 28 and 32 nucleotides of size. Then, rRNAs were removed, the RNA circularized and sequenced. To exclude a transcriptional regulation of the identified RP targets we performed a GE analysis from the same cell lines. Three independent experiments were performed for both approaches to look for statistically significant genes. As for the RP, we decided to conduct our subsequent analyses by taking in consideration those targets showing a Fold Change > 3.0. This cutoff allowed us to identify 1974 differentially regulated RPFs in SDOS-eGFP and 1779 in TRAP1-eGFP cells respectively. Besides, the GE analysis showed 78 and 738 differentially expressed genes (DEGs) upon SDOS-eGFP and TRAP1-eGFP overexpression. Then, we normalized the RPFs by ruling out the genes also present in the differential GE analysis, in order to select only those targets regulated at translational level. SDOS-targets normalization let us to identify 13 genes regulated both at transcriptional and translational level (Fig. 8A). As for TRAP1, the overlap between the RP and the GE lists showed that 97 genes are regulated at both level (Fig. 8A). Interestingly 209 RPFs resulted upregulated in both SDOS and TRAP1 RP libraries and, similarly, 254 were downregulated in both (Fig. 8B).

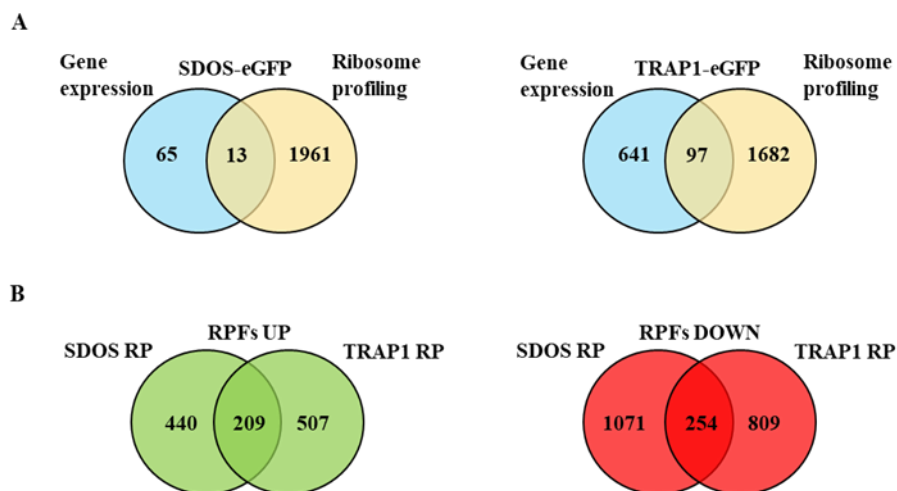


Figure 8 SDOS and TRAP1 are mostly involved in a translational regulation of specific substrates. (A) Venn diagrams showing SDOS-eGFP and TRAP1-eGFP RP and GE data in order to normalize differentially expressed RPFs. (B) Diagrams showing the overlapping of SDOS-eGFP and TRAP1-eGFP RP data.

4.6.3. Differentially expressed genes analysis

To look for selective pathways perturbed at gene expression level by SDOS and TRAP1, we performed a GO analysis on the above-mentioned GE data. Analysis for DEGs upon SDOS-eGFP overexpression showed, among the top downregulated biological pathways, enrichment in genes involved in cytoplasmic translation, translational termination, large ribosomal subunit, structural constituent of ribosomes and mitochondrial translation, strongly supporting our data about the influence of SDOS in mRNA translation (Fig. 9A). Considering that preliminary data from our group demonstrated that TRAP1 influence not only cytoplasmic translation - as already described - but also mitochondrial translation, it will be interesting to verify if SDOS might play a role in this process together with TRAP1 or if it might be fundamental for TRAP1-dependent mitochondrial translation regulation. Moreover, among the top downregulated pathways we also found enrichment in NADH dehydrogenase activity and amide biosynthetic process-associated genes (Fig. 9A), in agreement with the metabolic pathways-enriched

genes identified by iCLIP analysis, supporting a role for SDOS in the regulation of cell metabolism that could be orchestrated together with TRAP1. Analysis of the top upregulated biological pathways showed, among the others, enrichment in genes involved in nucleus localization, according to SDOS nuclear localization and its role in 53BP1-dependent repair pathway⁸⁶, and centrosome cycle (Fig. 9A). Centrosome is linked to cilia, since they represent distinct functional states of the same organelle, thus supporting the evidence that SDOS-bound transcripts are associated with ciliopathies. As for TRAP1, GO analysis of DEGs showed enrichment in genes associated to respiratory chain, oxidative phosphorylation, mitochondrial electron transport chain and cellular respiration among the top downregulated biological pathways (Fig. 9B). This evidence strongly supported our data about a TRAP1-dependent metabolic rewiring in ovarian cancer⁶⁹ and, more in general, its role in the regulation of cell metabolism, even if it remains to elucidate whether this regulation has an oncogenic or oncosuppressive potential⁷⁹. Besides, among the top upregulated biological pathways, we found enrichment in genes involved in ribosomal assembly and intrinsic apoptotic signaling pathway in response to endoplasmic reticulum stress response (Fig. 9B). Accordingly, we previously demonstrated that TRAP1 regulation of protein synthesis allows the synthesis of selective stress responsive proteins, such as ATF4 and Bip, thus providing protection against ER stress⁷⁴.



Figure 9 SDOS and TRAP1 gene expression data analysis confirm their functional role. (A) and (B) Analyses of biological pathways from SDOS-eGFP and TRAP1-eGFP GE data through GSEA. DEGs with p-values <0.05 were retained.

4.7. SDOS directly binds and regulates translation of transcripts of genes involved in primary cilia formation

iCLIP, RP and GE analyses produced a very high amount of data. Therefore, it was necessary to intersect the distinct results to have a global picture and to be able to select the most interesting regulated pathways associated to the proteins of interest. In this context, we decided to focus our downstream analyses on SDOS at first, since, as mentioned above, its functions are largely unknown. SDOS-eGFP iCLIP and RP data intersection identified a subset of 386 common genes. Analysis of diseases-associated genes showed, among the others, Joubert syndrome and Meckel Gruber syndrome, both belonging to ciliopathies family (Fig. 10A). In particular, TMEM67, CC2D2A and KIF7 were reported as genes associated to these diseases (Fig. 10B). Accordingly, cellular component-associated genes to the given subset showed enrichment in cilium-associated genes (Fig. 10C). Among the others, the above mentioned CC2D2A, TMEM67, KIF7 plus TMEM107 and TOPORS, all of them also known to cause ciliopathies⁹⁴. Taken together, these analyses were promising, since a new area of research is recently emerging linking cilia and centrosome proteins to DNA-damage response (DDR)⁹⁶, a process for which only recently it was described a role for SDOS⁸⁶.

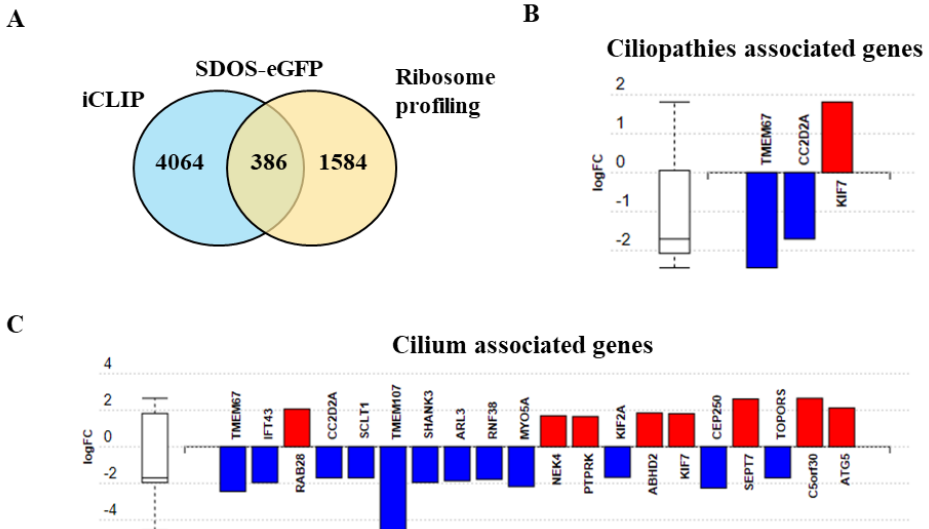


Figure 10 SDOS-directly regulated targets are involved in ciliopathies. (A) Venn diagram showing the number of SDOS-eGFP targets identified by iCLIP and RP. (B) and (C) Diseases-associated and cellular component-associated genes identified by using Advaita Bio's iPathwayGuide.

Starting from this evidence, among the iCLIP and RP common subset, we decided to validate those genes involved in cilia formation that were previously associated to ciliopathies: AHI1, CC2D2A, KIF7, NPHP1, RPGRIP1L, TCTN1, TMEM67, TMEM107, TOPORS, TTC21B and ZNF423. To confirm that these targets were bound at RNA level by SDOS as suggested by iCLIP-seq, we performed IPs of SDOS-eGFP and unfused eGFP followed by RNA extraction and qPCR, to measure the enrichment of targets mRNA in SDOS-eGFP compared to the eGFP negative control. In particular, among the selected group of genes, we focused on transcripts found at least in 2 out of 3 iCLIP libraries. Results confirmed that, among the others, TMEM107, CC2D2A and KIF7 mRNAs were significantly enriched in the SDOS-eGFP IP, while no enrichment was observed for ACTIN mRNA used as negative control (Fig. 11A). In order to confirm that all these genes were not differentially expressed upon SDOS overexpression, as suggested by GE data, we performed a qPCR analysis from SDOS-eGFP and unfused eGFP HeLa expressing cells. Results showed no changes at transcriptional level following SDOS-eGFP overexpression for all the selected genes but KIF7 (Fig 11B). Finally, we have begun to validate by WB the translation regulation of those targets present in the RP-seq data, either upon SDOS-eGFP overexpression or SDOS silencing. As shown in Fig. 11C, WB analysis of TMEM107 confirmed its down-regulation upon SDOS-eGFP overexpression, consistently with RP-seq. Accordingly, SDOS silencing caused an increase in TMEM107 protein level.

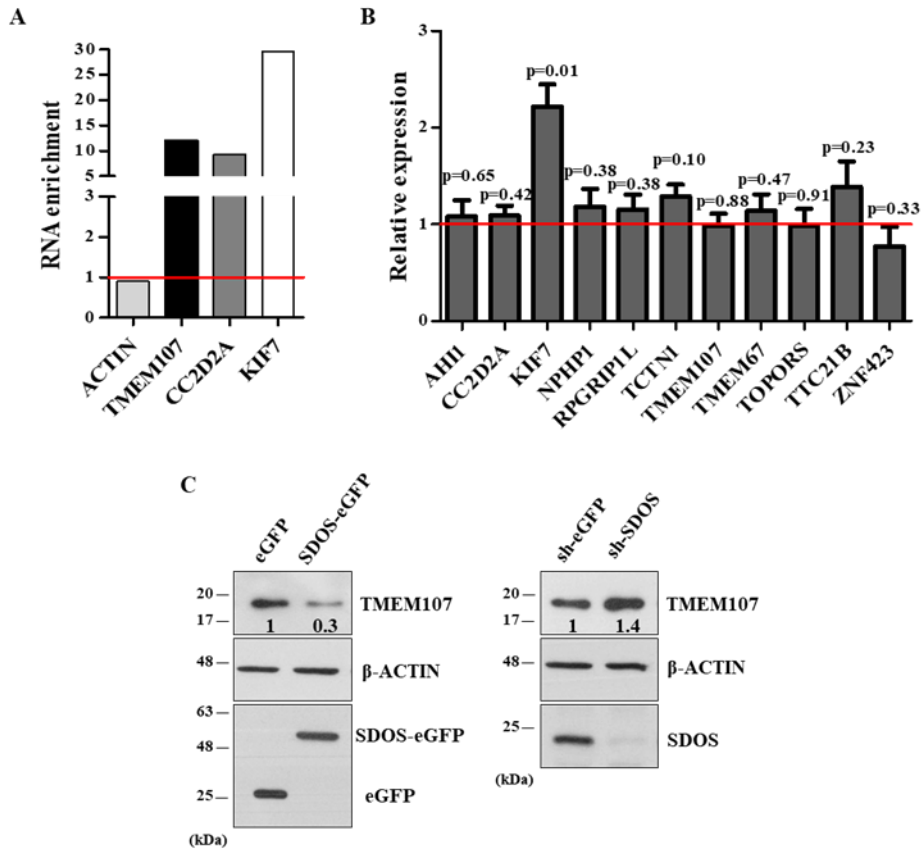


Figure 11 SDOS is an RBP which binds mRNA of genes involved in primary cilia formation and regulates their translation. (A) Validation of iCLIP results by RT-PCR of 3 biological replicates in RNA-immunoprecipitation experiments following both SDOS-eGFP or eGFP IP and RNA extraction. RNA enrichment in SDOS-eGFP IP relative to the eGFP control of specific substrates was normalized to a spike-in control (dashed line). ACTIN was used as negative control showing no enrichment. (B) RT-PCR of a subset of 11 genes responsible for ciliopathies from HeLa SDOS-eGFP and eGFP cells upon 24 hrs of induction. Data are expressed as mean \pm S.E.M. from four independent experiments with technical triplicate each. Numbers above bars indicate the statistical significance (P-value), based on one-sample t-test. Red line indicates expression level of the relative control. (C) WB analysis following SDOS overexpression or silencing with the indicated antibodies. Numbers indicate densitometric band intensities, calculated by assuming protein levels of the control equal 1.

5. DISCUSSION

Post-transcriptional events represent a complex and intricate layer of gene expression regulation. Accordingly, many players are required to fine tune these processes, allowing cells to adapt to internal or external stimuli when needed. Among the others, RBPs are emerging as key players in the regulation of each step of RNA life, from splicing and polyadenylation to transport, localization, translation and degradation¹¹. Given their fundamental role in these processes, the interest in RBPs has grown over the years with hundreds of novel RBPs recently identified that lack the classical RBDs identified so far, therefore known as “non-canonical” RBPs⁴. Considering that cancer cells strongly rely on post-transcriptional mechanisms for the re-programming of gene expression to be able to survive in critical conditions, it is not surprising that perturbations of RBP-associated networks can contribute to cancer development in several ways⁵². RBPs dysregulation in cancer arise from various mechanisms, including genomic alterations, transcriptional and post-transcriptional control, and post-translational modifications (PTMs). In particular, PTMs are the main responsible of RBPs dysfunction, with RNA-recognition elements susceptible to various PTMs thus influencing RBPs binding properties, function or localization⁵². Among the post-transcriptional mechanisms governed by RBPs intervention, translation is one of the most commonly dysregulated in cancer. Several studies demonstrated that all of the major oncogenic signaling pathways associated with cancer - PI3K/AKT/mTOR, RAS/MAPK, and Wnt/b-catenin – lead to dysregulation of translation⁴⁷. In this context, we investigated the role of two putative RBPs: the molecular chaperone TRAP1 and its putative partner SDOS.

TRAP1 was extensively described for its role in cancer through its anti-apoptotic and anti-oxidant functions associated with its mitochondrial localization^{97,98}. Very recently, a growing interest is emerging about the role of TRAP1 in the regulation of cancer cell metabolism that we demonstrated to be responsible for resistance to antitumoral drugs in ovarian cancer⁶⁹. However, our previous works helped to describe a more complex scenario, due to a newly identified localization of TRAP1 on the endoplasmic reticulum, linked to a specific function. Indeed, we demonstrated that TRAP1 is responsible for a co-translational regulation of specific substrates mostly directed

to mitochondria⁷². Moreover, TRAP1 associates with the translational apparatus, including both ribosomes and translational factors, thus influencing translation and affecting related features such as cell migration^{74,76}. In this context, the aim of this work is to shed further light about previously suggested RNA-binding properties of TRAP1⁸³ and its interacting-partner SDOS. SDOS was only recently described to have a role in cancer, by masking the histone methyl-lysine binding function of 53BP1 - with whom it forms a stable complex - thus influencing double-strand break repair⁸⁶. Our data strongly demonstrated that SDOS is a novel TRAP1-interacting partner and that this interaction occurs on the ER, supporting the hypothesis of a cooperation between SDOS and TRAP1 in translation. Accordingly, this work demonstrated that SDOS associates with ribosomes as well. Association with ribosomes often reflects an influence on the rate of translation which ensures correct translation and co-translational folding of newly synthesized proteins⁹⁹. Such regulation is relevant in cancer since dysregulated biosynthesis is one of the hallmark of cancer cells; moreover substrate-specific regulations can take place to enhance translation of proteins involved in carcinogenesis¹⁰⁰.

Polysome profiling following SDOS overexpression showed changes in the amount active polysomes relative to the control. This data was confirmed by monitoring of global protein synthesis through radioactive labelling of newly synthesized proteins which demonstrated that SDOS influence global protein synthesis. Moreover, SDOS is a paralog of NUDT16 proteins which are decapping enzymes, therefore involved in the formation of mRNPs aggregates to drive mRNAs degradation. Although SDOS lacks the decapping activity⁸⁴, component of SGs have been identified in this work by MS as putative SDOS partners, namely CCT4, CCT5 and CCT8⁸⁹. Therefore, we hypothesize that SDOS, differently from NUDT16, might take part to these mRNPs granules that are not anymore considered as site for mRNAs degradation but rather storage site for mRNAs sorting and processing, for either re-initiation of translation or degradation⁹⁰. Aberrant SGs formation contributes to neurodegenerative disease and some cancers¹⁰¹. Interestingly, we identified SDOS as a new component of SGs, where it co-localizes with polyadenylated RNAs upon sodium arsenite treatment, a well-known inducer of SGs, thus supporting a role for SDOS in the processing and sorting of specific mRNAs.

The most relevant finding of this work is the demonstration that both TRAP1 and SDOS are able to bind RNAs, result achieved by combining two highly specific approaches. Given the central role played by RBPs in mRNA translation, we hypothesize that the RNA-binding properties of both SDOS and TRAP1 might be relevant for their contribution to this process. The combination of three powerful high throughput techniques – iCLIP, Ribosome Profiling and differential Gene expression analysis – allowed us to demonstrate that TRAP1 and SDOS are, indeed, able to directly bind a subset of specific substrates at RNA level and to regulate them at translational level. However, SDOS-directly regulated targets did not extensively overlap those of TRAP1, suggesting that the two proteins have distinct properties although sharing some regulatory functions.

Analysis of TRAP1 data mostly confirmed its previously described role in metabolism and translation, raising questions about the existence of a cross-talk between these two processes. Accordingly, among the TRAP1-directly regulated targets that we identified, there are several proteins involved in the regulation of cell metabolism like UQCC1, UQCRC2, GFPT1 and PFKM among others.

Surprisingly, analyses of SDOS-directly regulated targets not only confirmed its possible role in translation but clearly indicated that most of these targets are responsible for ciliopathies, when mutated or dysregulated. In particular, Joubert Syndrome, Meckel Gruber syndrome and Retinitis pigmentosa are among the diseases associated to the gene set enriched in the SDOS target list. Ciliopathies belong to a class of rare diseases which are caused by defects in primary cilia⁹⁴. Primary cilia act as key coordinators of signaling pathways during development and in tissue homeostasis. These organelles were found in the majority of cells that are in G0 phase; when cells re-enter the cell cycle and start to divide, primary cilia are resorbed and grown again once the cells become quiescent¹⁰². Studies relative to cilia and cancer demonstrated that usually cancer cells display a reduction in the number of cilia, due to mechanisms involving the loss of genes required for ciliogenesis rather than altered proliferation rate¹⁰³. Accordingly, it was recently demonstrated that inhibition of ciliogenesis led to earlier tumor formation, faster tumor growth rate, higher tumor grade formation, and increased metastasis in breast cancer¹⁰⁴. Association of cilia with cancer is particularly interesting to further dissect the role of SDOS in cancer. Indeed, a new area of

research is exploring the possibility of a link between cilia and DNA-damage response⁹⁶, a process in which SDOS plays a fundamental role as recently described⁸⁶. Vertii A. *et al* suggested that the ATM co-factor ATMIN is not only involved in DNA damage but also acts a transcriptional regulator of ciliary DYNLL1, that we found as a SDOS protein partner by MS. In line with this evidence, we also found FXR2 among the transcripts bound by SDOS and upregulated among differentially expressed RPFs. FXR2 is an RBP - often co-deleted with p53 in some cancers¹⁰⁵ – that, according to bioinformatic analyses, might interact with ubiquitin C, an ubiquitin ligase, which regulates ARL6¹⁰⁶, a protein involved in membrane protein trafficking at the base of the ciliary organelle¹⁰⁷. Therefore, among the identified SDOS-directly regulated targets, we validated a subset of 11 genes whose dysregulation was previously demonstrated to cause ciliopathies. None of these genes is regulated at transcriptional level by SDOS, except KIF7. Among them TMEM107 was of particular interest because the snoRNA U8, that is the main target of NUDT16-decapping activity and whose recognition seems to be conserved by SDOS⁸⁴, is located in the 3'-UTR region of TMEM107. Of the selected subset of genes, we validated CC2D2A, KIF7 and TMEM107 as SDOS-bound transcripts, according to our iCLIP data. Moreover, we confirmed that, among the others, TMEM107 is regulated at translational level either upon SDOS overexpression or silencing. These data strongly support the idea of a role for SDOS in primary cilia formation and related ciliopathies or cancer. One possibility might be that SDOS/DYNLL1 interaction regulates SDOS binding to cilia-associated transcripts which, in turn, could be implicated in mRNAs transport in the proximity of cilia and localized translation, and this signaling could be associated with DDR.

Taken together, the data of this work strongly demonstrated that both SDOS and TRAP1 are involved in a translational regulation of specific substrates bound at RNA level that are involved in pathways often dysregulated in cancer, like metabolism and ciliogenesis among the others. Intriguingly, a link between cilia defects and metabolic dysfunction was already described in patient affected by obesity and type II diabetes¹⁰⁸, suggesting the existence of a possible cross-talk between these processes in cancer.

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8. LIST OF PUBLICATIONS

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Three years ago, I had the chance to choose between the opportunity to begin this adventure called PhD and another job. It was not so hard for me to choose back then, because I was sure and convinced more than ever. I thought a lot about that moment of my life during these years, and it always remind me about the famous film “Sliding Doors”. I used to picture what would have happened if I had “lost” that train and my life would have taken a different road. In the hardest moments of this incredible, crazy, enthusiastic, sometimes frustrating, stimulating, long, devious, funny, unforgettable path, I pictured that my life would have probably been more “easy”, more satisfying. And yet, while I am here in my room reading my PhD thesis for the millionth time, looking for every possible mistake, I found hard to describe my feelings, and I cannot stop thinking that I would make that choice over and over again. I had the chance to meet lot of people along this road which I need to thank, because each one of them teach me something that I will never forget.

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Being a scientist can make you feel alone most of the time, it is not easy to let people into this world and to easily explain what it means to do research. Not to mentioned that failures are more frequent than victories in our world. But, when that tiny victory arrives, it brings with it something capable of wipe away months of frustrations and anger, which reminds you the reasons why you choose to be part of that world. It reminds you the will of doing something good. That’s why, despite everything, I cannot think of a more beautiful job.

Tre anni fa ho avuto la possibilità di scegliere tra il cominciare questa avventura chiamata dottorato ed un altro lavoro. Non ebbi bisogno di molto tempo per decidere, ero più convinto e determinato che mai. In questi tre anni ho spesso ripensato a quel momento della mia vita che richiama alla mia memoria il famoso film "Sliding Doors". Ho spesso immaginato cosa sarebbe successo se avessi "perso" quel treno e la mia vita avesse preso una piega diversa. Nei momenti più difficili di questo incredibile, pazzo, entusiasmante, a tratti frustrante, stimolante, lungo, tortuoso, divertente, indimenticabile percorso, ho immaginato che quella stessa vita sarebbe stata probabilmente più "facile", che mi avrebbe regalato più soddisfazioni. Eppure oggi, mentre sono nella mia stanza e rileggo per la milionesima volta la mia tesi alla ricerca di errori vari ed eventuali, provo un'emozione difficile da descrivere e non posso fare a meno di pensare che rifarei quella stessa scelta, sempre. Tante sono le persone che ho incrociato lungo questo percorso e che sento il dovere di ringraziare, perché chi più, chi meno, tutte mi hanno insegnato qualcosa che non dimenticherò mai.

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Fare il ricercatore può farti sentire solo il più delle volte, perché non è facile lasciar entrare le persone in questo mondo, riuscire a spiegare con parole semplici cosa significhi fare ricerca. Per non parlare del fatto che i fallimenti sono molto più frequenti delle vittorie nel nostro mondo. Eppure, quando quella piccola vittoria arriva, è in grado di spazzare via mesi di frustrazioni e rabbia, e riporta a galla i motivi che ti hanno spinto a sceglierlo, quel mestiere. Riporta alla luce la voglia

di fare qualcosa di buono. Ed è per questo che, nonostante tutto, non riesco ad immaginare un lavoro più bello.